EXTRACTED SUGAR-BEET PULP AND SUCROSE, TWO RENEWABLE MATERIALS AS “HOT” SUBSTRATES FOR ENZYMATIC SYNTHESIS OF VALUABLE SACCHARIDES

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)
genehmigte
D i s s e r t a t i o n

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eingereicht am: 01.04.2004
mündliche Prüfung (Disputation) am: 17.06.2004
2005
(Druckjahr)

Die vorliegende Arbeit entstand während meiner Tätigkeit als wissenschaftliche Mitarbeiterin an der Abteilung Technologie der Kohlenhydrate der Technischen Universität Braunschweig im Zeitraum von Oktober 2000 bis Oktober 2003. Diese Arbeit wurde im Rahmen der industriellen Gemeinschaftsforschung und des von der Deutschen Forschungsgemeinschaft geförderten Projekts „Screening neuer Glycosyltransferasen zur Produktion von Oligosacchariden“ innerhalb des DFG Sonderforschungsbereichs 578 "Integration gen- verfahrentechnischer Methoden zur Entwicklung biotechnologischer Prozesse – Vom Gen zum Produkt“ unter der Leitung von Herrn Prof. Dr. Klaus Buchholz durchgeführt.

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Gemeinsamen Naturwissenschaftlichen Fakultät, vertreten durch den Mentor, in folgenden Beiträgen vorab veröffentlicht:

Publikationen:

Baciu, I., Berensmeier, S., Jördening, H.-J. & Buchholz, K. Gewinnung von Galacturonsäure aus Zellwandbestandteilen der Rübenschnitzel. *Zucker Industrie* 11, 127, (52): 845-853 (2002).

Baciu, I.-E. & Jördening, H.-J. Kinetics of galacturonic formation from sugar-beet pulp. *Enzyme and Microbial Technology*. In press.

Baciu, I.-E., Jördening, H.-J., Seibel, J. & Buchholz, K. Investigations of the transfructosylation reaction by fructosyltransferase from *B. subtilis* NCIMB 11871 for the synthesis of sucrose analogue galactosyl-fructoside. *Journal of Biotechnology*. Submitted.

Baciu, I.-E., Beine, R., Kicinska, A.-M., Wittrock, S., Bode, W., Hofer, B. & Buchholz, K. Characterisation of different wilde type and recombinant dextransucrases with respect to new substrates and oligosaccharide synthesis. In Vorbereitung.

Tagungsbeiträge:

Baciu, I.-E., Tadiggs, T., Jördening, H.-J. & Buchholz, K. Wertstoffgewinnung aus extrahierten Rübenschnitzeln: Thermisch/enzymatische Behandlung zur Produktion von Galacturonsäure. *(Vortrag). 19. DECHEMA Jahrestagung der Biotechnologen*, Leipzig (2001).

Baciu, I.-E., Jördening, H.-J. & Buchholz, K. Kinetics of the enzymatic production of galacturonic acid. *(Poster) The 5th International Symposium on Biocatalysis and Biotransformation Biotrans 2001*, Darmstadt (2001).

Baciu, I.-E., Jördening, H.-J. & Buchholz, K. Gewinnung von Galacturonsäure aus pektinhaltigen Rohstoffen. *(Poster) GVC/ DECHEMA Jahrestagungen 2002 mit 20. Jahrestagung der Biotechnologen*, Wiesbaden (2002).

Baciu, I.-E., Jördening, H.-J. & Buchholz, K. Production of galacturonic acid from the cell-wall of sugar-beet pulp. *(Poster) 15th International Congress of Chemical and Process Engineering CHISA 2002*, Czechien (2002).

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Baciu, I.-E., Jördening, H.-J. & Buchholz K. Screening neuer Glycosyltransferasen zur Produktion von Oligosacchariden. *(Poster) 5. Veranstaltung der Vortragsreihe "Angewandte Umweltbiotechnologie aus Niedersachsen" der Fachgruppe Umwelt des Life-Science Netzwerkes Niedersachsen: Biokonversion nachwachsender Rohstoffe*, Braunschweig (2003).

ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude to Professor K. Buchholz, the coordinator of this thesis, for offering me the opportunity to accomplish my Ph. D. degree, for his support and involved guidance of my research.

I express my thanks to Prof. Dr.-Ing. D. Hempel for accepting to review this thesis as first examiner.

I am especially indebted to my supervisor and second examiner, Privatdozent Dr. H.-J. Jördening for his constant constructive support, valuable discussions and thoughtfulness, critical expertise of this thesis, which contributed to the fulfilment of my work.

I appreciatively acknowledge also Dr. T. Taddigs and Mrs. N. Thielecke for their collaboration to the studies regarding the exo-polygalacturonase screening, galacturonic acid separation and concentration.

I am thankful as well to the collaborators for their efforts and helpful discussions: to Dipl. Biotechnol. Marko Malten (electrophoresis), Dr. Wang (isoelectrical focussing), Dr. Moser (FTF purification with ion exchange resins), Dr. Hofer (dextransucrase from *S. oralis*), Mrs. Scherbard (scanning electron microscopy) and Dipl. Chem. Raluca Niesner (microscopy).

The author thanks to Nordzucker InnoCenter, Novo Nordisk and Herbstreith & Fox for providing the extracted sugar-beet pulp, some enzyme preparations, respectively the sugar-beet pectin. My appreciation is extended to Nordzucker InnoCenter as well for offering several enzyme preparations, but also for allowing me to carry out the cell culture experiments.

I owe also many thanks to Amino GmbH for placing the preparative chromatographic system at my disposal and as well for support by experience.

For the pleasant working environment and warm-hearted assistance, I am grateful to all my colleagues and collaborators from Technology for Carbohydrates, Nordzucker InnoCenter and Amino GmbH, especially to Sonja Berensmeier, the only biotechnologist in our group and Anne Marquardt.

Last, but not at least I am deeply grateful to my family, especially to my husband, who encouraged and helped me to keep my perspectives.

Acknowledgements

The research project “Production of galacturonic acid from extracted sugar-beet pulp” (AiF-Project No. 12020 N) was financially supported by FEI (Forschungskreis Ernährungsindustrie e.v., Bonn, Germany). Funds from the AiF and the Ministry of Economics and Technology are gratefully acknowledged.

“Microbial synthesis of sucrose analogue galactosyl-fructoside from sucrose” is a subproject (A3) of the SFB 578 joined project “Development of biotechnological processes by integrating genetic and engineering methods - From gene to product”. The financial support provided by DFG (Germany) is appreciatively acknowledged.

CONTENTS

ABBREVIATIONS AND SYMBOLS

Act.	Activity
AF	Arabinofuranosidase
Ara-Fru	Arabinosyl-fructoside (arabinosucrose)
β Gal	β-Galactosidase
Bx°	Refractometric index (Brix degrees)
Conc.	Concentration
CP	Citrus pectin
DS	Dextransucrase
DTE	Dithioerythritol
DTT	Dithiothreitol
E_a	Activation energy
EA	Endo-arabinanase
Endo-Gal	Endo-galactanase
Endo-PG	Endo-polygalacturonase
ESB	Extracted sugar-beet pulp
Exo-PG	Exo-polygalacturonase
FFF	Filtrate concentrated by frontal flow rate ultrafiltration
Fru	Fructose
FOS	Fructooligosaccharides
FTaF	Filtrate concentrated by tangential flow rate ultrafiltration
FTF	Fructosyltransferase
GA	Galacturonic acid
Gal	Galactose
Gal-Fru	Galactosyl-fructoside (galactosyl-fructose, galsucrose)
Gal-Fru substrate solution	Represents the end reaction solution with inactivated FTF, which contains residual sucrose, galactose, Gal-Fru, glu-

	cose and fructose
Glu	Glucose
GOS	Glucooligosaccharides
GTF	Glycosyltransferase
IC	Ion chromatography
IEC	Ion exchange chromatography
IEF	Isoelectric focusing
IMO	Isomaltooligosaccharides
Inj. peak	Injection peak
kDa	10^3 Dalton
K'_{IP}	Apparent product inhibition constant (mmol/L)
K'_{IS}	Apparent substrate inhibition constant (mmol/L)
K'_{M}	Apparent Michaelis-Menten constant (mmol/L)
$K'_{M\ CP}$	Apparent Michaelis-Menten constant for citrus pectin (mmol/L)
$K'_{M\ ESB}$	Apparent Michaelis-Menten constant for extracted sugar-beet pulp (mmol/L)
$K'_{M\ SBP}$	Apparent Michaelis-Menten constant for sugar-beet pectin (mmol/L)
Man-Fru	Mannosyl-fructoside (mannosucrose)
Mel	Melibiose
MWCO	Molecular weight cutt-off
OD_{580}	Optical density at λ=580 nm
OS	Oligosaccharides
[P]	Product concentration (mmol/L)
PAE	Pectin acetyl esterase
PC	Pressure control unit
PG	Polygalacturonase
PGA	Polygalacturonic acid

PL	Endo-pectin lyase (Endo-pectin transeliminase)
PME	Pectin methyl esterase
r	Initial reaction rate [mmol/(L x min)]
R_f	Response factor
RFF	Retentate concentrated by frontal flow rate ultrafiltration
RG	Rhamnogalacturonan hydrolase
RGAE	Rhamnogalacturonan acetyl esterase
RGL	Rhamnogalacturonan lyase
r_{max}	Maximal reaction rate [mmol/(L x min)]
rpm	Rounds per minute
RTaF	Retentate concentrated by tangential flow rate ultrafiltration
[S]	Substrate concentration (mmol/L)
SBP	Sugar-beet pectin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sta	Stachyose
TLC	Thin layer chromatography
TC	Temperature control unit
U	Enzyme units
Xyl-Fru	Xylosyl-fructoside (xylsucrose)

1. INTRODUCTION

Use of biocatalysts for the conversion of renewable resources

The biocatalysts or enzymes, which can rightly be called the catalytic machinery of living systems, have been indirectly used since the beginning of mankind history. Historical discoveries revealed that the biotechnology is older than written history, dating back as far as 4000 years before Christ when malting and fermentation were practiced in Mesopotamia [1]. Accordingly, the first applications of enzymes were in food manufacturing, for cheese and bread making, vinegar, beer, wine and whiskey production.

Isolated enzymes were firstly used in detergents in the year 1914 by the Röhm company (Germany) and their large-scale production started in the early 1960s [2]. Since then, the biocatalyst field has developed with a rapid rate and has conquered new areas of application: food, cosmetic, pharmaceutical, diagnostics, textiles, agriculture, animal feed and polymers leading to a wide variety of product types. The estimated value of world enzyme market is presently about US $ 1.3 billion and it has been forecasted to grow to almost US $ 2 billion by 2005. Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) are the main industries, which use about 75% of industrially produced enzymes [2].

The rapid growth of biocatalysis is the result of research and development in two key technologies: protein engineering, including molecular evolution and enzyme engineering. While the first provides enzymes with altered structure, function and selectivity, particularly in aqueous media, the second involves engineering of the enzyme microenvironment, leading to improvements in non-aqueous media and non-classical environments (gas-phase and supercritical fluids). Consequently, biocatalytic processes can be carried out in organic solvents and the applications range from chiral resolution of pharmaceuticals and chemicals to enantio- and regioselective polymerisation [3]. The combination of genetic techniques, protein engineering and computer modelling will yield enzymes with tailored selectivities and synthetically relevant activities in any reaction medium. Consequently, the chemical production by enzymes and living cells (whole-cell biocatalysts) will be furthermore directed to green alternative synthetic pathways.

During the last decades, the research in the area of renewable materials has shown that the main biological building-blocks of life, proteins, fibres, fats, carbohydrates and their derivatives can successfully substitute and in many cases outperform products from

traditional non-renewable sources. In addition, renewable materials ensure environmental advantages, because the bio-processes are generally related to reduced emissions and the final products are biodegradable and recyclable. Therefore, in the last years the use of renewable resources has grown progressively and they have become one of the key-targets for biocatalysis, as response to the increasing demand of different industrial branches (chemical/pharmaceutical, food and paper-making) to environmental friendly applications, in agreement with the green chemistry standards. Today, the range of product sectors where biological “renewables” can play a role is steadily growing and includes building materials, textiles, paper, bulk and fine chemicals, plastics, foodstuffs and various energy sources. Furthermore, biotechnology can provide the appropriate tools for increased yield, functionality and purity of bioproducts and can facilitate the production of tailor-made renewable materials suitable to cover an endless range of applications [4].

Until today, some by-products of the food industry were not taken into consideration as supply and precursors for valuable and novel, interesting substances. However, the development of sugar technology and the significant growth of the biocatalysts field have initiated new strategies for the exploitation of by-products. Among them, in the spotlight of recent research stand the by-product sugar-beet pulp and the source material, sucrose as representative, desired low-priced “hot substrates” for the production of high-priced compounds.

Carbohydrates are by far the most abundant organic compounds on earth and represent the major proportion of the annually renewable global biomass of about 200 billion tons. Up to now, only 3% of this quantity are used by man, the rest being decayed and recycled along natural pathways [5]. Carbohydrates present outstanding properties which enable their use both for chemical and biochemical transformations. Their polyfunctionality ensures the performing of chemical synthesises, whereas their hydrophilic character and solubility in water secure biocompatibility and biodegradability. Consequently, innovative approaches could be realised by integrated biotechnological and chemical processing. Actual and expected trends for sucrose and sucrose derivatives encompass a fascinating range of products such as: oligomers as building blocks for polymers, surfactants, paint formulation, new adhesives and pesticides. Therefore, this vast unexploited potential made the carbohydrates exceptionally appropriate candidates for industrial application as chemical raw materials.

1.1. Galacturonic acid formation from extracted sugar-beet pulp

Sugar-beet pulp and pellets represent a suitable cheap renewable resource for the production of valuable pectin compounds such as galacturonic acid. However, up to now there are no technical methods available, which could be used for this purpose. Therefore the aim of this work is the developing of an integrated system of processes, including new enzymatic agents, which should be able to fulfil this demand.

Extracted sugar-beet pulp represents the main by-product of the industrial extraction of sugar-beet (*Beta vulgaris*). This renewable material is produced annually in massive quantities and presents the advantage to be available during all the year, not only in campaign (from september to december). These two aspects make extracted sugar-beet pulp a potential source of commercial pectin, due to its high content of this type of polysaccharides (15-25%).

The composition of extracted sugar-beet pulp, according to *Oosterveld* [6], is schematic illustrated in Fig. 1.

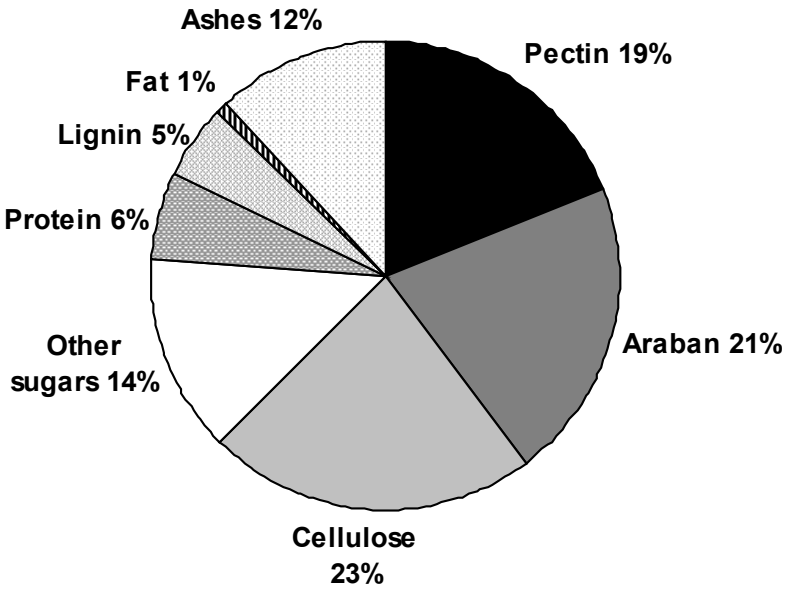


Fig. 1: Composition of the extracted sugar-beet pulp [6]

Until today sugar-beet pulp is mainly used in cattle feeding [6], but it is also suitable as dietary fibre source [7].

Pectin substances are complex mixtures of polysaccharides, located in the cell-wall-middle lamella complex and contribute both to the adhesion between the cells and to the mechanical strength of the cell-wall, behaving in the manner of stabilized gels [8]. From this point of view, pectins (from apple and citrus) are mainly used as a gelling agent and less importantly as a texturizer, stabilizer, thickener and emulsifier in food.

Pectins are consisting of a backbone of α-(1-4)-linked galacturonic acid residues, partially esterified (with methyl groups: 7.5–75.5%) [9] forming long "smooth" regions which may be interrupted by "hairy" regions, which contain mainly neutral sugars.

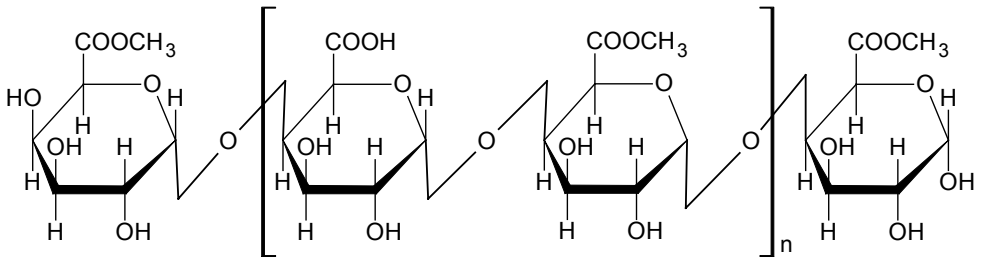


Fig. 2: "Smooth" region structure: polymer of galacturonic acid which is partially esterified

The "hairy" regions consist of three subunits which can be present in different ratios. Firstly, a rhamnogalacturonan subunit, consisting of a backbone of alternating α-(1-4)-linked galacturonic acid and α-(1-2)-linked rhamnose residues, partially substituted with single galactose residues β-(1-4)-linked to the rhamnose residues. The second subunit, also a rhamnogalacturonan is substituted with long araban side-chains, in which the arabinose residues are mainly terminally (1-3)-linked and (1-3,5)-linked. The presence of the third subunit, a xylogalacturonan subunit has been proven to exist (in apple) and consists of a galacturonan backbone, 3-substituted with single xylose residues [6]. The schematic structure of apple pectin is exemplified in Fig. 3.

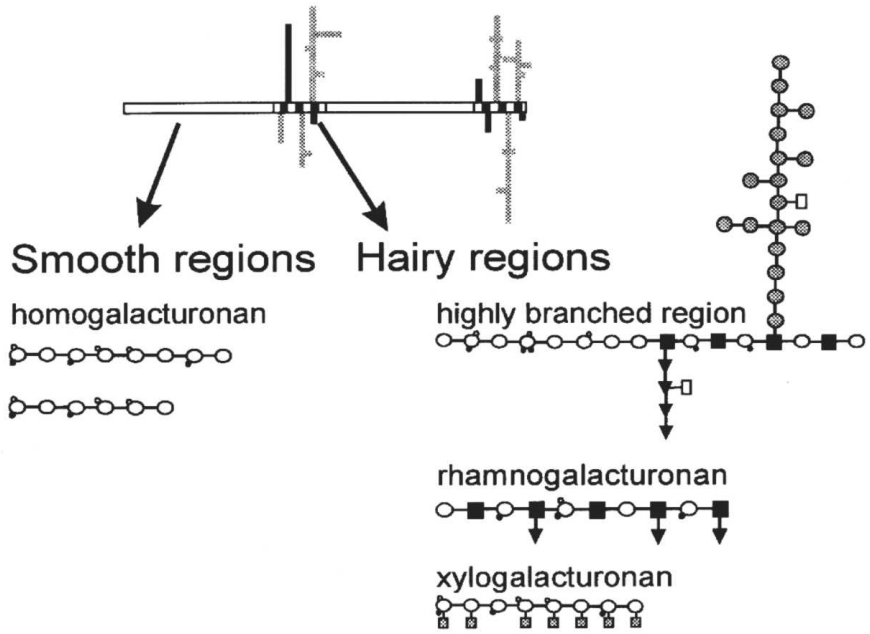


Fig. 3: Schematic structure of apple pectin: ▼ galactose; ○ galacturonic acid; ■ rhamnose; ● arabinose; ■ xylose; □ ferulic acid; • acetyl group; ° methyl ester [6]

Beet pectin differ from other pectins (apple or citrus) by its high rhamnose content, by the presence of acetyl groups linked to the α-D-galacturonic acid and by the presence of ferulic acid (0.5-1%), one of the major phenolic acids of plant cell walls. It is attached to the O-2 position of (1-5)-linked arabinose residues in the araban chains and to the O-6 position of galactose residues in (1-4)-linked galactans.

The contents of pectin compounds are: galacturonic acid accounts for 54.4-77.9%, the rhamnose for 0.9-3.2%, arabinose for 1.8-12.5% and galactose for 2.4-8.1%. The presence of acetyl groups in pectins extracted from sugar-beet inhibits gelling properties, the main functional feature of apple and citrus pectins, but enhances the emulsifying properties [10].

Pectinolytic enzymes are produced by plant pathogenic fungi and bacteria and are useful enzymes in fruit juices and wines fabrication (extraction, clarification and liquefaction) as also in fabric and papermaking industry [11].

The enzymes active towards the “smooth” homogalacturonic regions are pectin methyl esterase (PME), pectin acetyl esterase (PAE), endo-polygalacturonase (endo-PG) and endo-pectin lyase or endo-pectin transeliminase (endo-PL).

The rhamnogalacturonan hydrolase (RG), lyase (RGL) and acetyl esterase (RGAE), arabinofuranosidase (AF), endo-arabinanase (EA), endo-galactanase (endo-Gal) and β-

galactosidase (β Gal) "attack" the "hairy" regions. The exo-polygalacturonase (exo-PG) is active towards both regions [6]. Polygalacturonase (PG) hydrolyses only demethylated pectin and therefore additionally requires the action of PME and PAE. The enumerated enzymes and their site of action are indicated in Fig. 4.

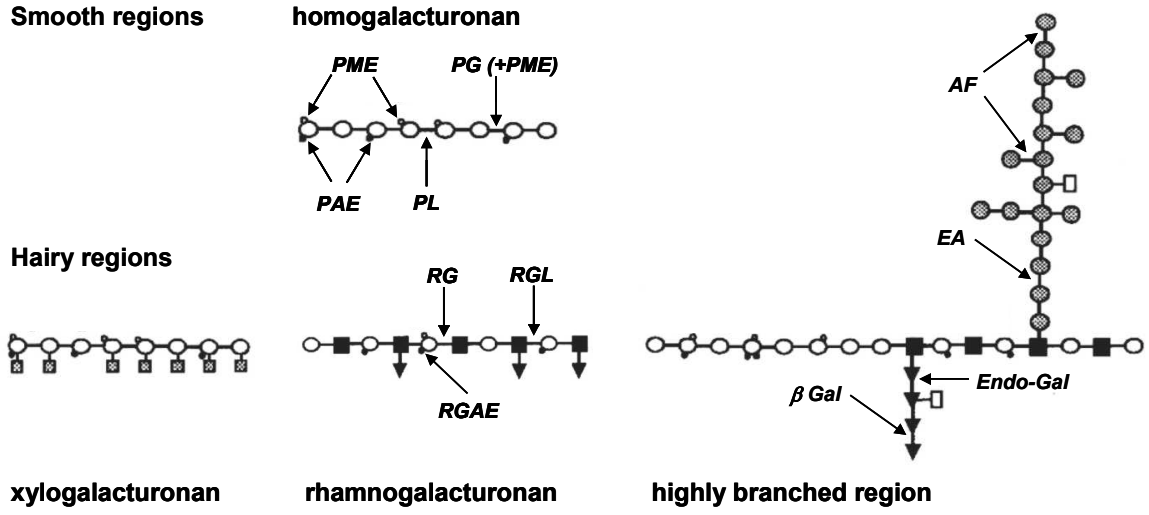


Fig. 4: Enzymes active on "smooth" and "hairy" regions of pectins (▼ galactose; ○ galacturonic acid; ■ rhamnose; ● arabinose; ■ xylose; □ ferulic acid; • acetyl group; ° methyl ester) [6]

Latest studies demonstrated that the dietary pectin reduces significantly the levels of total cholesterol and low density lipoprotein cholesterol [12]. It is also demonstrated that pectin substances lower blood glucose and insulin levels and also participate in weight reduction. Pectin is newly a component in haemostatic formulations and in manufacturing of encapsulated pharmaceuticals [8].

The building blocks of pectin are also of economical interest. For example, ferulic acid which chemically resembles vanillin, can be biotransformed in flavouring compounds, as well as rhamnose, through chemical pathways (for caramel, roasted and fruit flavours). L-arabinose can be used for diagnostic purposes in bacteriology and has anti-Parkinson properties [10]. Galacturonic acid can be utilised for the synthesis of tensioactive agents by esterification with various fatty acid for pharmaceutical and cosmetic purposes [13] - [15].

1.2. Microbial synthesis of sucrose analogue galactosyl-fructoside from sucrose

Oligosaccharides (OS) have been commercialised since the 1980's as alternative sweeteners. More recently, they gained much interest, as building blocks in pharmaceuticals and, as response to an increasing demand from the consumer for healthier and calorie controlled foods, also as compounds in the modern, so called "functional food". There are several prebiotic OS on the European and US market, such as the inulin type fructans, fructooligosaccharides (FOS), isomaltooligosaccharides (IMO) [16] and galactooligosaccharides. Functional food, firstly introduced in Japan (Japan being actually the leader in prebiotic food development), has no universally accepted definition, but can be described as food or food ingredients that may provide health benefits and prevent diseases [17].

New alternative sweeteners on this basis are important not only because of their sweetness, but also because of their functional properties. Some of the desirable characteristics of FOS are: low caloric and safe sugars for diabetics from the fact that they are barely degraded by the digestive enzymes and utilised as an energy source in the human body, at a reduced level. They are also non-cariogenic and not metabolised by oral *Streptococci* to form acids and insoluble glucan, which are the cause of the dental caries [18].

Such non digestible food ingredients are better known under the name **prebiotics**, that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria of the microflora in the colon (indigenous *Bifidobacterium* and *Lactobacillus* genera), which are generically named **probiotics** [19]. In view to attain a two-fold beneficial effect, the pro- and prebiotics were combined in so- called **synbiotics**. Consequently, synbiotics are defined as mixtures of pro- with prebiotics which influence positively the well-being of the host by supporting the survival of probiotics and the colonisation of the colon with beneficial bacteria [20].

FOS are found naturally in many foods, such as wheat, asparagus, onions, sugar-beet, Jerusalem artichoke and chicory, plants from which they can be isolated. A range of prebiotics, precisely α-glucooligosaccharides (GOS) are manufactured by enzymatic synthesis with glucosyltransferase (GTF) [21].

Today, FOS mixtures are produced commercially on large scale either by controlled hydrolysis of inulin or via synthesis from sucrose or lactose as raw material with fructosyltransferases (FTFs) [22]. All these oligosaccharides are marketed for human

nutritional application as food complements, in combination with specific microbial flora and vitamins and also as additives in dermocosmetic products [21].

GTFs have been subjected to an intensive research in the last three decades, mainly those involved in industrial dextran production, such as dextransucrase (DS) and alternansucrase produced by *Leuconostoc sp*. In spite of the growing importance of FOS in food industry, the FTFs are much less studied compared to GTFs, fact which represented the challenge for starting this work.

FTFs can have two origins: firstly, they can be obtained from plants (mentioned above) and secondly, from microorganisms such as fungi (*Aspergillus sp., Penicillium sp.*), yeasts (*Saccharomyces cerevisiae, Aureobasidum sp.*) and bacteria (*Bacillus sp., Streptococcus salivarius, Arthrobacter sp.*).

Concerning the transfructosylation properties, the FTFs can be classified in two families, following the CAZy database classification [23]:

1. **Glycoside Hydrolase Family 32 (GH 32)** which encompasses:

1.1 Fructans and FOS synthesising enzymes originating from plants:

1.1.1 1-FFT: 1,2-β-Fructan 1F-fructosyltransferase (EC 2.4.1.100)

1.1.2 1-SST: sucrose 1F-fructosyltransferase (EC 2.4.1.99)

1.2 Bacterial fructan synthesising FTFs from *Lactobacillus* and *Streptoccocus sp.*:

1.2.1 Fructosyltransferase: FTF (Aldose β-D-fructosyltransferase EC 2.4.1.162) produced by *Bacillus subtilis NCIMB 11871, 11872, 11873*, with a high propensity to form a wide range of β-(2-1) linked disaccharides, so called sucrose analogues, but which does not form polymer [24]

1.2.2 Invertase (β-fructofuranosidase) (EC 3.2.1.26) shows also transfructosylation activity when acting on high sucrose concentrations, producing as well FOS (the invertase obtained from *Aspergillus sp.*, *Penicillium sp.*, *Saccharomyces cerevisiae*), as also sucrose analogues [the invertase from *Arthrobacter sp. K-1* (FEPM BP-3192)] [25]

2. **Glycoside Hydrolase Family 68 (GH 68)** that includes:

2.1 Levansucrase: LS (EC 2.4.1.10) catalyses the synthesis of levan, a β-(2-6)-linked fructose homopolymer with 5-20% β-(2-1) branching points, from sucrose.

Levansucrases are produced by *Aerobacter levanicum, Aureobasidium sp., Bacillus subtilis, Erwinia sp., Pseudomonas sp., Rahnella aquatilis, Streptococcus mutans, Zymomonas mobilis* [26]. Levansucrases from *B. subtilis* [27], *Erwinia sp.* [24] and *Aerobacter levanicum* [28] have also been used to synthesise sucrose analogues such as galsucrose (Gal-Fru), xylsucrose (Xyl-Fru), mannosucrose (Man-Fru) and arabinosucrose (Ara-Fru).

2.2 Invertase (β-fructofuranosidase) (EC 3.2.1.26) as already described.

As already mentioned, the sucrose analogues as approached in this thesis are β-(2-1)-α-linked disaccharides. As the name defines they represent substances with a similar structure to sucrose, in which the glucosyl residue from sucrose is substituted by an other aldosyl residue, for example galactosyl, in the case of galactosyl-fructoside (Gal-Fru). Thus, they are distinguishing from sucrose only by the arrangement of one hydroxylic group, in the case of (Gal-Fru) by the axial arrangement of the OH-group in the 4-position.

The sucrose analogues synthesis studied in this thesis occurs in the presence of *B. subtilis* FTF (EC 2.4.1.162), which transfers the fructosyl residue of the substrate sucrose (Suc) to the acceptor monosaccharide, galactose (Gal), as depicted in Fig. 5.

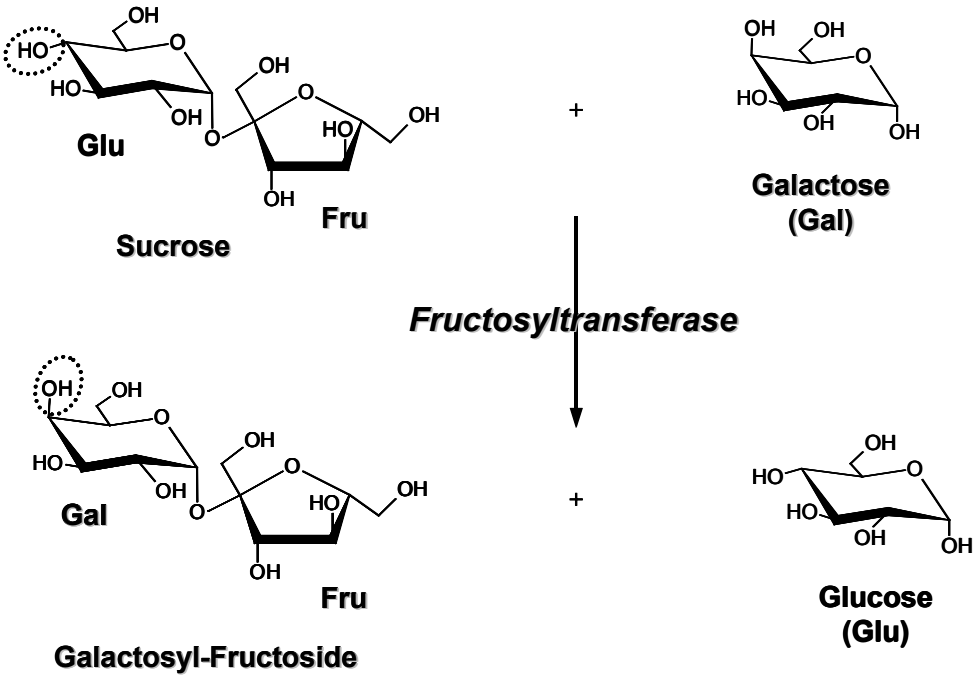


Fig. 5: FTF catalysed reaction for the synthesis of sucrose analogue galactosyl-fructoside

Not only sucrose, but also two other primary oligosaccharides, superior homologues of sucrose, raffinose (Raf) and stachyose (Sta) represent substrates for the FTF. The structure of these substrates is shown in Fig. 6.

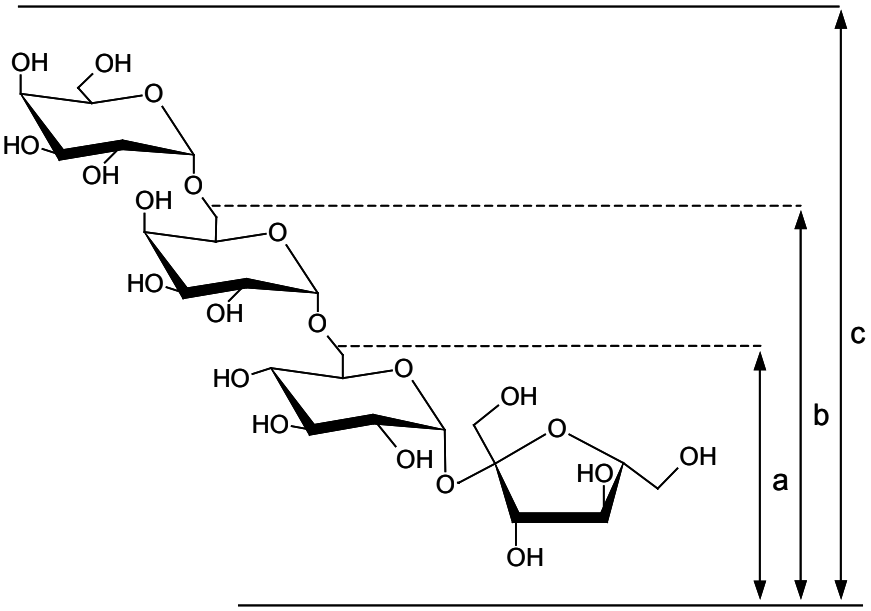


Fig. 6: Raffinose series of oligosaccharides: a – sucrose, b – raffinose, c – stachyose

The sucrose analogue synthesis reactions with raffinose and stachyose as substrates are very interesting from the scientific point of view, but at present of no economical interest, on the fact that the costs of these substrates are rather high compared to sucrose.

1.3. Aim of this thesis

The goal of this thesis is the use of biocatalysts as tools for the conversion of renewable materials in order to synthesise rare, high-priced saccharides. With this purpose in view, the research was focussed on the subsequent two topics:

a. Production of galacturonic acid, an interesting and valuable building block for pharmaceuticals, cosmetics and food ingredients (57 euro per 25 g galacturonic acid, Riedel de Haen, Germany, catalogue price) from extracted sugar-beet pulp, for an efficient valorisation of this by-product of the sugar refining industry.

b. Synthesis of a rare disaccharide, represented by the sucrose analogue galactosyl-fructoside (Gal-Fru) by using sucrose as substrate and

monosaccharide galactose as acceptor in the transfructosylation reaction catalysed by FTF from *B. subtilis*.

Essential aspects of this integrated multidisciplinary research approach are reaction engineering and analytical procedures as follows:

- enzyme screening for selecting from several potential candidates of the most suitable two enzymes for the aimed goals
- establishing optimal reaction conditions for both high yield and concentration of galacturonic acid and galactosyl-fructoside
- separation of the target products from the saccharide mixtures, in order to provide galacturonic acid and Gal-Fru on preparative scale
- kinetic characterisation of the galacturonic acid synthesis by degradation of extracted sugar-beet pulp
- analysis of the structure of Gal-Fru and the nature of the glycosidic bond
- FTF characterisation

These aspects are essential, since the yield of products depends on reaction engineering, as previously has been shown for several oligosaccharides synthetic routes before [29] - [32]. Furthermore, the presented tasks provide the data which are required for the rational design of feasible biotechnological processes.

2. EXPERIMENTAL SET-UP

2.1. Exo-polygalacturonase screening

The following comercial enzyme preparations were screened for exo-polygalacturonase activity: Gamapect press and Gamapect plus from Gama company (Germany), Pectinex® 100L and Pectinex® ULTRA-SPL from Novo Nordisk (Germany) and Rohapect D5L Special, Rohapect MPE and Rohament PL from Röhm (Germany). For all enzymes, a standard assay was performed as follows: 100 µL enzyme solution were added to 100 mL of 8 g/L citrus pectin substrate solution in sodium acetate-acetic acid buffer with a pH of 4.2, the reaction being performed at 50°C.

The galacturonic acid formation was studied by discontinuously taking aliquots at time intervals up to 7 h. The enzymes were inactivated by boiling the samples in a water-bath for ten minutes. After cooling, the inactivated samples were filtered through a 0.45 µm nitrocellulose membrane filter (Millipore, Eschborn, Germany) and analysed by means of HPAEC after appropriate dilution.

One 1 U exo-polygalacturonase (exo-PG) represents the volume of Pectinex® 100L (in mL) that releases reducing sugar equivalent to 1 µmol galacturonic acid (GA) per minute and mL substrate solution, under assay conditions.

2.2. Pectin extraction from sugar-beet pulp

Prior to the enzymatic attack with Pectinex® 100L, 4 g pressed extracted sugar-beet pulp were subjected to the treatment with 100 mL acidic, basic reagents solutions (acetic, citric, formic, sulphuric acid and respectively sodium hydroxide) and water in order to extract pectin. The investigation series was performed by heating at temperatures in a range from 95°C to 145°C for different time intervals (from 30 up to 390 minutes). At high temperatures (130°C and 145°C) the tests were carried out in an autoclave, so the pulp was additionally handled under pressure.

After the extraction procedure, enzymatic reactions were performed with the resulting product mixture consisting of pectin extract and pulp residues.

2.3. Separation and concentrating of the galacturonic acid

Separation of galacturonic acid from the reaction mixture was performed with the polystyrene-divinylbenzene sulphonate copolymer resin Finex CS13 GC in Na^+ form purchased by Finex™, Finland. Finex CS13 GC is a strongly acidic cation exchange resin with an average bead size of 100-200 µm and a 6% cross-linking degree.

50 mL filtered reaction solution, obtained after 24 h reaction catalysed by Pectinex® 100L was concentrated 20-fold by evaporation under reduced pressure. The 2.5 mL concentrate was loaded onto the resin (about 650 mL hydrated resin packed in a 1 m glass column from Kranich, Germany) and was eluted with water. Each separation step was followed by regeneration and washing of the resin with 1 M sodium hydroxide solution and water respectively. The separation was investigated at four pH values: 2.2, 3.2, 4.2 and 5.2.

A strong basic anion exchange resin Lewatit Monoplus M 500 in acetate form (Bayer AG, Germany) presenting quaternary ammonium groups and having a bead size of 600 µm, was selected as stationary phase for the concentrating of the galacturonic acid. The regeneration and washing steps of the 20 g resin packed onto a glass column were carried out with 1 M acetic acid and water respectively. The fractions resulted after the separation with Finex CS13 GC in Na^+ form (as above described) which contained mainly galacturonic acid (circa 5 g/L) were pooled, given on the Lewatit Monoplus M 500 column and then eluted. Three eluents were tested for this purpose: 2 M sodium hydroxide solution, phosphate buffer (pH 8.1) and Tris-HCl buffer (pH 9.1).

2.4. Substrates for Pectinex® 100L characterisation

As reference substrates industrial citrus pectin (CP) (74-80% galacturonic acid, grade of esterification 63-66%, M_r 30.000-100.000 from Fluka, Germany) and sugar-beet pectin (SBP: 70-75% galacturonic acid, grade of esterification 60%, M_r 50.000-150.000 from Herbstreith & Fox KG, Germany) were used.

Extracted sugar-beet pulp (ESB) (30% dry substance, 8-16% galacturonic acid), pressed (from a German sugar factory) and frozen for storage, was suspended in water and heated in water-bath at 95°C with magnetic stirring for one hour, in order to obtain the soluble pectin [33]. The enzymatic reactions were performed with the resulting mixture (pectin extract and pulp residues).

2.5. Enzyme mixture Pectinex® 100L

Pectinex®100L is a highly active, pectinolitic enzyme mixture, which was produced by a selected strain of *Aspergillus niger*. It contains mainly pectin transeliminase, polygalacturonase and pectin esterase, as well as small amounts of hemicellulase and cellulase. It is used mainly in the food industry for a rapid depectinisation, for the degradation of fruits and vegetables and the production of apple and pear juices [34].

The enzyme of interest, the polygalacturonase, catalyses the hydrolytic cleavage of the O-glycosyl bond of α-(1-4)-polygalacturonan. There are two main types of polygalacturonases. Exo-polygalacturonase (exo-PG) remove uronic acids residues in a terminal fashion, from the non-reducing end of the pectin chains and endo-polygalacturonase (endo-PG) which cleaves the α-(1-4)-glycosidic linkages randomly along the uronide chain. Both types of enzymes require low methoxylated pectin [35], [36]. Pectin methylesterase removes methyl ester groups from the polysaccharide in a random fashion. The demethylated product is the substrate for polygalacturonase (and pectatlyase) [37].

Pectinex®100L was delivered with a standard activity of 5000 $U^{55°C}$/mL. After 60 minutes incubation, the enzyme mix was very stable at 40°C, showed a loss of 15% activity at 50°C and of 80% at 60°C in buffered solutions with a pH of 3.5. These both activity and stability tests were measured by the depectinisation of apple juice, performed by the Novo Nordisk and described in the delivery note [34].

2.6. Effect of pH on Pectinex® 100L activity

The effect of pH on the exo-PG of Pectinex® 100L activity toward polygalacturonic acid (PGA - 1 g/L) was examined in the pH range 3 - 5.4 in sodium acetate-acetic acid buffer at 50°C.

2.7. Effect of NaCl and $CaCl_2$ on Pectinex® 100L activity

The influence of these salts, respectively cations on the enzyme activity were investigated by adding 0.1 M NaCl, 0.4 mM $CaCl_2$ and a mixture of both salts to 6 g/L sugar-beet pectin and 1 mL (57.8 U), respectively 2 mL (115.6 U) Pectinex® 100L in sodium acetate-acetic acid buffer at pH 4 and 50°C.

2.8. Pectin kinetics

Substrate kinetics experiments were carried out with citrus pectin solutions of 1, 3, 6, 8, 12, 15, 20, 25 and 50 g/L, with sugar-beet pectin in concentrations of 3, 5, 6, 8 and 12 g/L and with extracted sugar-beet pulp (dry weighed) in amounts of 2, 5, 10, 20, 30, 40, 50, 75, 100, 150, 200, 250 and 300 g/L.

2.9. Galacturonic acid inhibition

Two experimental series were performed with citrus pectin as substrate and galacturonic acid (product) already added at the start of the reaction.

In the first series, the same amount of galacturonic acid (final concentration of 6 g/L) was added to the citrus pectin solutions of 3, 6, 8 and 12 g/L.

The second series of reactions was carried out with different amounts of galacturonic acid (6, 9 and 12 g/L) added to 8 g/L substrate solution.

2.10. Mathematical methods

For the determination of kinetic constants both linearisation procedures and a numerical integration method were used.

Concerning the linearisation methods, the Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf transformations of the Michaelis-Menten equation were used for first calculations of the K'_M and the r_{max} values [38].

- Lineweaver-Burk:

$$\frac{1}{r} = \frac{K'_M}{(r_{\max} \times [S])} + \frac{1}{r_{\max}} \quad (1)$$

- Hanes-Woolf:

$$\frac{[S]}{r} = \frac{K'_M}{r_{\max}} + \frac{[S]}{r_{\max}} \quad (2)$$

- Eadie-Hofstee:

$$-\frac{1}{r} = \frac{r_{\max}}{K'_M} + \frac{r'}{K'_M} \quad (3)$$

Further calculations were then performed with a commercial available integration program "Modelmaker 3.03" developed by the Cherwell Scientific Publishing Ltd. The differential equation was integrated by the fourth-order Runge-Kutta method. An adaptive step-size control mechanism was used. A simplex algorithm for the iterative optimisation of parameters and start concentrations was used. Thereby the Chi-square, as sum of normalized error squares, was minimised [39].

2.11. Fructosyltransferase screening

Several enzymes and enzyme preparations were subjected to the screening, with the system 40% sucrose (refined sugar - Raffinade, EG Qualität I, Nordzucker, Germany) and 40% acceptor, at optimal conditions for each enzyme. Galactose, mannose and xylose (ICN Biomedicals, Germany) were tested as acceptors.

Fructosyltransferase (FTF - EC 2.4.1.162) was produced by fermentation of the *B. subtilis* NCIMB 11871 strain, obtained from the National Collection of Industrial and Marine Bacteria, United Kingdom [24]. Invertase and Pectinex ULTRA-SPL [40], [41] were provided from Roche Diagnostics GmbH (Germany) and Novo Nordisk (Germany) respectively. The enzyme preparations containing endo-FTF (*Aureobasidium sp*.) [42], levanfructotransferase (*Arthrobacter ureafaciens* K2032) [43] and levansucrase (EC 2.4.1.10 from *Zymomonas mobilis* ZM1 ATCC 10988) were kindly offered by Nordzucker InnoCenter (Braunschweig, Germany).

2.12. Bacterial strain, cultivation and harvesting of the cells

For the culture growth of *B. subtilis* NCIMB 11871 a liquid mineral salts medium containing 1% sucrose (w/v) as carbon and energy source was used as described by *Cheetam et al.* [24], [44]. The FTF activity was also tested by cultivating the strain on the same medium enriched with 0.1% (w/v) yeast extract. The mineral salt medium [45] had the following composition as summarised in Tab. 1.

B.subtilis NCIMB 11871 strain was maintained on nutrient agar plates consisting of the medium described above supplemented with 15 g/L agar (as regularly for general solid growth medium) at 4°C and subcultured two times monthly at 30°C for 48 h to maintain viability.

Tab. 1: Mineral salt medium composition

Salt	Concentration [mg/100 mL]
KH_2PO_4	136.0
$Na_2HPO_4 \times 2H_2O$	267.0
$(NH_4)_2SO_4$	50.0
$MgSO_4 \times 7H_2O$	20.0
$CaCl_2 \times 2H_2O$	1.0
$FeSO_4 \times 7H_20$	0.5
$MnSO_4 \times H_2O$	0.18
$Na_2MoO_4 \times 2H_2O$	0.25

The inoculum development stage consisted of three successive transfers as required for activation of the culture and to build sufficient volume for inoculation for the final production medium. 50 mL seed flasks, containing 5 mL sterile minimal sucrose medium, were inoculated from an agar plate culture of *B.subtilis* NCIMB 11871. After 12 h growth at 30°C and under shaking at 150 rpm, the 5 mL of the first culture were used to inoculate second stage seed flasks. The 5 mL inoculum was added to 100 mL of fresh sterile minimal sucrose medium in 500 mL seed flasks and grown as described for the first stage inoculum development. After 12 h of growth, the entire content of one flask was then used to inoculate one litre sterile minimal sucrose medium. This main culture was carried out under shaking (150 rpm) at 30°C for 48 h. At periodic intervals, samples were taken for biomass growth and FTF activity determination. Biomass growth was measured spectometrically by the absorbance at 580 nm (OD_{580}) using a Shimadzu Spectrophotometer UV-120-02, having distilled water as reference (blank).

2.13. Fructosyltransferase supernatant separation

In the stationary phase, the cells were harvested by centrifugal separation at 5000 × g for 15 minutes at 4°C (SORVAL® Centrifuge Rotor SS 34, GSA and GS-3 DuPont, USA) and then discarded. The supernatant obtained was assayed undiluted, as crude enzyme solution for the characterisation but also as concentrated solution (the crude enzyme solution was subjected to a purification treatment by utilising known purification methods such as ultrafiltration). The supernatant was stored at 4°C and to prevent bacterial contamination, both 0.02% NaN_3 and 1% toluene were added.

2.14. Fructosyltransferase assay

One unit FTF was defined as the amount of enzyme required to release 1 µmol of

reducing sugar equivalent to glucose per minute under assay conditions.

A reaction mixture containing 40% (w/v) sucrose (1169 mmol/L) as substrate and 40% (w/v) galactose (2222 mmol/L) as acceptor were prepared in 2.5 mL Sörensen phosphate buffer (pH 6) by magnetic stirring and heating at 50°C for solubilisation for 30 minutes in a water bath. Sucrose was the main substrate employed, but also raffinose and stachyose were tested for substrate specificity.

After incubating at the temperature chosen, the reaction was started by adding the equivalent volume of FTF supernatant (circa 6 U FTF/2.5 mL supernatant) (obtained as described in section 2.13). The sucrose analogue formation was investigated by discontinuously taking aliquots at suitable time intervals up to 48 h.

The enzyme was inactivated by boiling the samples in a water-bath for ten minutes. After cooling, the inactivated samples were filtered through a 0.22 µm nitrocellulose membrane filter (Millipore, Eschborn, Germany) and analysed, after appropriate dilution. Analysis of the samples was carried out using several chromatographic systems (section 2.21).

2.15. Protein techniques

The SDS-PAGE, N-terminal sequencing and binding tests were performed in collaboration with the research group of Prof. Dr. Jahn (Institute of Microbiology, TU-Braunschweig). Isoelectric focussing was carried out at GBF mBH (Braunschweig, Germany) in the collaborating working group of Prof. Dr. Deckwer.

2.15.1. Protein determination

Protein content was determined as described by Bradford assay [46] using bovine serum albumin (Albumin fraction V, Merck, Germany) as protein standard, ranging from 10 to 100 µg per 100 µL phosphate buffer (pH 7) and phosphate buffer as blank. The absorbance was measured at 595 nm with a SHIMADZU UV-1201V spectrophotometer with an accuracy of 2%.

2.15.2. Concentrating by ultrafiltration

Two ultrafiltration processes were tested: frontal and tangential flow rate.

Frontal flow rate ultrafiltration was performed on a filtration cell Vivacell 250 (Vivascience, Germany) at pressure values between 2-4 bar. Two polyethersulfone membranes with low

proteic adsorption, of 30 and 50 kDa MWCO (**M**olecular **W**eight **C**utt-**O**ff) (Vivacell 250 insert, Vivascience, Germany) were used.

Ultrafiltration with tangential (cross-over) flow rate was carried out with a Filtron Mini-Ultrasette module (Pall Gelman Laboratories, Germany) equipped with 50 cm^2 low protein binding OMEGA™ polyethersulfone membrane, with 30 kDa MWCO. A Watson-Marlow pump ensured a retentate and filtrate flow of 600 mL/min, respectively of 6 mL/min.

With both methods, 100 mL FTF supernatant was subjected to a 10-fold concentrating. The resulted 10 mL retentate and 90 mL filtrate were afterwards tested for FTF activity and protein content as above described.

2.15.3. Electrophoresis

Several FTF supernatants resulted in two fermentation series and the supernatants from the FTF purification test series were subjected to electrophoresis. 3 mL of each were sterile centrifuged at 5000 rpm for 15 minutes at room temperature. Afterwards, a volume of 2.5 mL centrifuged supernatant was given on a Sephadex G-25 column (Pharmacia, Germany), the run through volume was discarded and the proteins were eluted with 3.5 mL TE-buffer. 1.75 mL of the eluted samples were additionally concentrated by evaporation for 12 h at 40°C in a vacuum concentrator. The concentrated samples were next treated with 7 µL bidistilled water and 3 µL SDS-buffer, boiled for ten minutes at 100°C and after cooling, spotted on gels.

The electrophoresis analysis of the samples obtained by purification of FTF by using ion exchange resins was performed with crude (not concentrated) solutions.

SDS-PAGE was then performed according to *Laemmli* [47] by using the Ana-phor system (Anamed, Germany) with 12% acrylamide slab gel (Anamed, Germany). Proteins were silver stained and as protein markers SDS-PAGE high-molecular-mass standards from 31 to 250 kDa were used (Prestained Protein marker Broad range, BioLabs Inc., England).

2.15.4. N-terminal amino acid sequencing

In order to determine the N-terminal amino sequence, the gels resulted by SDS-PAGE electrophoresis were electroblotted onto a polyvinylidene difluoride (PVDF) membrane and subjected to amino acid sequence analysis by Edman degradation. The search in EMBL and SWISS-PROT databases was performed using Blast and Profile search programs.

2.15.5. 2 D gel-electrophoresis

Precipitation of proteins

10 mL of the 30 kDa RTaF (RTaF defines the fructosyltransferase **R**etentate concentrated by **Ta**ngential **F**low rate ultrafiltration) were treated with 20% trichloracetic acid (w/v) and 0.1% DTT (dithiothreitol) (w/v), thoroughly mixed and stored to precipitate for 15 h at 4-8°C. The resulted precipitate was afterwards subjected to centrifugation at 13.000 rpm and 4°C (Hereaus, Biofuge fresco, Germany). After the removal of supernatant, the pellet was re-suspended in minus 20°C cold washing buffer 0.1% DTT in acetone (w/v), stored for 30 minutes at minus 20°C and then centrifuged as above described. The washing step was repeated for two times, for an efficient recovery of proteins. At the end of the washing procedure, the pellet was dried for 15 minutes by using a vacuum concentrator. The dehydrated pellet was resolubilised in 4.8 mL rehydration buffer (8 M urea and 0.1% TritonX-100).

First dimension: isoelectrical focussing

A volume of 350 µL rehydrated pellet was spotted on a 18 cm IPG pH 3-10 stripe (Amersham Pharmacia, Germany) and the program summarised in Tab. 2 was applied.

Until performing of second dimension, the stripe was stored at minus 80°C, followed by the treatment with equilibration buffer: 50 mM Tris-HCl pH 6.8, 6 M urea, 30% glycerin (v/v), 2% SDS (w/v) and 1% DTE (w/v). All chemicals were of highest purity available from commercial sources.

Tab. 2: Isoelectric focussing program

Step Nr.	Voltage [V]	Time [h]
1	30	6
2	60	6
3	200	1
4	500	1
5	1000	1
6	1000-8000	0.5 (gradient)
7	8000	8

Second dimension: SDS-PAGE

The electrophoretic separation of the proteins was carried out as described in section 2.15.3, without concentrating of the samples.

2.15.6. Purification of fructosyltransferase with ion exchange resins

These tests were carried out in the working group of Dr. Moser from the Institute of Microbiology, TU-Braunschweig.

One type of cation exchange resin, CM Sepharose Fast Flow (Pharmacia, Germany) and two types of anion exchange resins, DEAE-52 Cellulose (Whatman, Germany) and DEAE Sepharose Fast Flow (Pharmacia, Germany) were tested with regard to their capacity to bind FTF.

The investigations were carried out for three pH values: pH 5 (citric acid-sodium citrate buffer), pH 6 (MES buffer) and pH 8.1 (HEPES buffer).

For the removal of fine particles, 100 mg of each ion exchange resin were washed several times with 1 M appropriate buffer, until the wanted pH value was achieved and after that centrifuged. The equilibration of the ion exchange carriers occured in two steps, by washing with 20 mM corresponding buffer.

10 mL of 30 kDa RTaF was dialysed prior binding for 12 h against 50 mM MES buffer (Serva, Germany). Subsequently, 1 mL of the dialysed 30 kDa RTaF was added to 110 µL of appropriate buffer and mixed with 100 µL equilibrated ion exchange material (at the corresponding pH value). The binding took place for 15 minutes at 4°C, then the ion exchange resin was centrifuged and washed with 1 mL of 20 mM appropriate buffer.

Proteins were next detected by SDS-PAGE electrophoresis. 12 µL of 30 kDa RTaF, dialysed 30 kDa RTaF and of the centrifugates after binding were subjected to the procedure as described in section 2.15.3, the proteins being revealed with Coomassie Brilliant Blue R-250 (Sigma, Germany).

2.16. pH and temperature influence on fructosyltransferase activity

The influence of pH on FTF activity was examined by measuring the amount of glucose released in buffer systems at pH ranging from 3 to 8 (in citric acid-sodium citrate buffer) at 30°C. For the determination of the optimal temperature, the FTF activity was investigated in the temperature range from 20 to 60°C at a pH value of 6 in Sörensen phosphate

buffer. All tests were carried out as described in section 2.14.

2.17. Fructosyltransferase reaction series

Standard conditions for activity tests were as given in 2.14. Three kinetics series with different concentrations of substrate and acceptor were carried out as follows:

- 20% sucrose (584 mmol/L) and 5, 10, 20, 40% galactose (278, 556, 1111, 2222 mmol/L)
- 40% sucrose (1169 mmol/L) and 15 , 30, 60% galactose (833, 1667, 3333 mmol/L)
- 40% galactose (2222 mmol/L) and 5, 10, 20, 40 and 60% sucrose (146, 292, 584, 1169 mmol/L)

Moreover, the hydrolysis of sucrose was investigated in a test series with 30% sucrose (877 mmol/L) with the FTF supernatant and two retentates resulted by concentrating using frontal flow ultrafiltration: 30 kDa RFF and 50 kDa RFF (RFF represents the fructosyltransferase **R**etentate concentrated by **F**rontal **F**low).

2.18. Immobilisation procedure of fructosyltransferase

For this purpose, three immobilising materials were tested: Trisopor®-Amino and Eupergit®, in 2 commercial forms: Eupergit® C and Eupergit® C 250 L. Trisopor® and Eupergit® carriers were gifts from Schuller (Germany) [48], respectively Röhm Pharma (Germany) [49].

Trisopor®-Amino was activated (at room temperature) as follows: 3 g carrier (particle size of 100-200 µm, pore size of 100 nm $\pm$ 5, pore volume 1105 mm^3/g and specific surface of 42.99 m^2/g) were suspended in 25 mL citrate-phosphate buffer to ensure a pH of 4.5, then for five minutes degassed *in vacuo*. After degassing and filtering, the carrier was mixed with 15 mL of 5% glutaraldehyde solution (in citrate-phosphate buffer pH 4.5), allowing to react for 30 minutes, at ambient pressure under gentle shaking. Activated Trisopor® was washed in two successive steps with distilled water (20 mL for each washing step) and afterwards stored at 4°C, until use, in the appropriate buffer solution (citrate-phosphate buffer pH 4.5), containing 0.02% NaN_3 and 1% toluene.

For immobilisation, 0.3 g (wet weight) activated Trisopor® was mixed with 2 mL of 30 kDa RTaF (1 g wet weight Trisopor®/6.66 mL 30 kDa RTaF) and 1 mL of 1 M Na_2SO_4, then

incubated under shaking at room temperature. After 24 h, the carrier was washed two times with Sörensen phosphate buffer of pH 6 (10 mL for each washing step), subsequently stored and preserved prior use, as above described.

The immobilisation procedure for both types of Eupergit® (average particle size of 200 μm, approx. pore diameter of 100 nm and specific surface of 53 m^2/g) was the same: 3 mL of 30 kDa RTaF, was brought to pH 6, with 1 M Sörensen phosphate buffer, then 0.5 g Eupergit® was added (1 g wet weight Eupergit®/6 mL 30 kDa RTaF). The immobilisation was carried out under gentle shaking by incubating for 24 h in a water bath, at 25°C. After incubation, the solution was separated by filtration through 12 μm nitrocellulose membrane filter (Millipore, Eschborn, Germany). The beads were washed in three repetitive steps with Sörensen phosphate buffer pH 6 (10 mL for each washing step). The immobilised enzyme was stored in buffered solution (pH 6), containing 0.02% NaN_3 and 1% toluene, at 4°C.

The reactions were performed with 2 mL substrate-acceptor solution (40% sucrose-40% galactose) and started by adding the equivalent wet weight of immobilisate to 2 mL of 30 kDa RTaF (30 kDa RTaF/immobilisate wet weight ratio of 1/1), which represent approx. 0.3 g Trisopor®, 1.44 g Eupergit® C and 1.2 g Eupergit® C 250 L.

2.19. Galactosyl-fructoside structure determination

The structure of the Gal-Fru was determined both on enzymatic pathways and with analytical, as spectroscopic and polarimetric methods (which are described in section 2.21.5).

Invertase test

Invertase from *Saccharomyces cerevisiae* (Roche Diagnostics GmbH, Germany) was used for the product structure determination. In this view, the pH of the Gal-Fru substrate solution (represented by the end reaction solution with inactivated FTF, which contains residual sucrose, galactose, Gal-Fru, glucose and fructose) was adjusted to 4.6 and then thermostated at a temperature of 30°C. 10 U invertase per mL Gal-Fru substrate solution were added to start the reaction. Prior to addition, the invertase, which was provided in lyophilised form, was solubilised in the appropriate buffer: 167 U invertase (0.5 mg) per mL of 0.32 M citrate buffer with a pH of 4.6. The same preparation procedure was applied for the tests with pure Gal-Fru, with the distinction that 100 mg Gal-Fru (117.65 mg Gal-Fru of 85% purity) per 1 mL appropriate buffer were used. The reactions were also investigated by discontinuously sample taking at determined time intervals.

2.20. Sample preparation for preparative chromatography

Prior to preparative chromatography separation, the Gal-Fru substrate solution was subjected to an enzymatic treatment, on two pathways as below described.

a. The first treatment was performed with wild type dextransucrase (DS) from *Leuconostoc mesenteroides NRRL B 512 F* (Critt Bio-Industries, DGBA-INSA, Toulouse, France), immobilised in alginate beads [29].

   For the DS reaction, the Gal-Fru substrate solution was supplemented with 50 mg/L $CaCl_2$, then brought to a pH of 5.2 and 30°C. The reaction was started by adding 2 U immobilised DS/mL Gal-Fru substrate solution. After eight hours reaction, the reaction was stopped by filter separation of the alginate beads containing DS. The beads were after that washed with distilled water and stored until use at 4°C in 20 mM sodium acetate buffer of pH 5.2 with 50 mg/L $CaCl_2$.

   The solution obtained by filtering was next treated with an α-glucosidase (E-Sucrase, Megazyme International Ireland Ltd.). For this purpose, the pH of the solution was adjusted to 6.8, then the mixture was thermostated at 40°C and the reaction was started by adding 5 U α-glucosidase/mL reaction solution (after solubilisation of the lyophilised enzyme in Sörensen-phosphate buffer having a pH value of 6.8).

b. The second treatment of the Gal-Fru substrate solution was carried out with wild type DS from *Streptococcus oralis* cloned in *E. coli*, purchased by the collaborating working group of Dr. Hofer (GBF mbH, Germany)

   In this case, the pH of the Gal-Fru substrate solution was adjusted to 5.4, then the reaction solution was thermostated at 30°C. The enzymatic reaction was started by adding 1 U modified DS/mL Gal-Fru substrate solution.

2.21. Analytical methods

For qualitative and quantitative determinations, the analysis of the reaction products were performed with three chromatographic systems: Dionex-HPAEC (Dionex, Germany) with pulsed amperometric detection–HPAEC-PAD [50], [51], with an ion chromatograph (Metrohm, Germany) with UV/RI-detection and with a thin-layer chromatography system.

2.21.1. HPAEC-PAD: Dionex

Dionex-HPAEC consists of: pump: gradient pump module Dionex (Germany); flow: 1 mL/min.; autosampler: Basic Maraton Spark (Holland); chromatograph: Dionex (Germany); column: CarboPac PA1 4 × 250 mm (Dionex); guard column: Carbo PAC MA1 (Dionex); detector: PAD with gold electrode; temperature: 30°C; software: Chromeleon (Dionex, Germany).

The eluents used were A = 0.1 M NaOH and B = (0.1 M NaOH + 1 M CH_3COONa).

For the analysis of galacturonic acid and oligogalacturonides within 20 minutes the composition changes from 50% A and 50% B to 0% A and 100% B (program 4).

For sucrose-analogue, mono-, di- and oligosaccharides within 30 minutes the eluent mixture changes from 100% A and 0% B to 74% A and 26% B (program 7).

Standards for Dionex

The neutral monosaccharides (L-arabinose-Aldrich, Germany; D-fructose, D-galactose, D-glucose, D-mannose and D-xylose-ICN Biomedicals, USA and L-rhamnose-Sigma, Germany), the disaccharides sucrose (refined sugar-Raffinade, EG Qualität I, Nordzucker, Germany) and melibiose (ICN, Germany), the trisaccharides raffinose, 1-kestose (Fluka, Germany), the tetrasaccharide nystose (Fluka, Germany) and acidic sugars (D-galacturonic acid monohydrate-Riedel de Haen, Germany, digalacturonic and trigalacturonic acid-Sigma, Germany) were used as external standards for peak identification and quantification.

Standard solutions for calibration were prepared in the range of 0.1 and 1 g/L.

The retention times for galacturonic acid and galactooligouronides are as follows: arabinose 3.8 ± 0.2 min; galactose 4.5 ± 0.3 min; rhamnose 3.4 ± 0.3 min, galacturonic acid 12.2 ± 0.2 min, digalacturonic acid 15.0 ± 0.1 min and trigalacturonic acid 17.3 ± 0.1 min.

Mono-, di-, tri- and oligosaccharides have the following retention times: mannose 6.2 ± 0.2 min; galactose (Gal) 6.4 ± 0.2 min; glucose (Glu) 7.5 ± 0.3 min; fructose (Fru) 8.6 ± 0.3 min; Gal-Fru: 11.5 ± 0.3 min.; melibiose (Mel) 12.1 ± 0.2 min; sucrose 12.6 ± 0.3 min; raffinose (Raf) 13.7 ± 0.1 min; 1-kestose 14.2 ± 0.2 min and nystose 17.2 ± 0.4 min. The oligosaccharides emerge from a retention time higher than 18 minutes.

The relative standard deviation represents maximum 5%.

2.21.2. Ion chromatography: IC

The chromatographic system consists of: pump: IC-pump 697 (Metrohm, Germany); autosampler: 698 (Metrohm, Germany); chromatograph: ion chromatograph 690 (Metrohm, Germany); software: Gynkosoft Version 5.3 (Gynkotek, Germany). Two systems were used for the analysis of the carbohydrates as described below:

a. The analysis of galacturonic acid and galactouronides was performed with the first system consisting of: column: Wescan Anion Exclusion Column 300 x 7.8 mm, Alltech; guard column: Phenomenex® Carbo-H^+ (Phenomenex, Germany); eluent: 2 mM H_2SO_4; detector: UV-detector Lambda 1000 (Bischhof, Germany) at room temperature.

b. For the FTF educts and products a second system was used: eluent: bidistilled water; flow: 0.8 mL/min; column: RCM Monosaccharide Ca^{2+} 300 x 7.8 mm, (Phenomenex®, Germany); detector: refractive index (RI) detector ERC-7512 (Erma Inc., Germany). The column has a working temperature of 80°C and is temperated with a Thermasphere™ TS-130 oven (Phenomenex®, Germany).

Standards for IC

Standard solutions for IC calibration were prepared furthermore with the saccharides used for the HPAEC-PAD system and with the tetrasaccharide stachyose (Sta) (ICN, Germany), in the range of 0.1 and 10 g/L.

Retention times of the tetra-, tri-, di- and monosaccharides are for the first and second system as follows: galacturonic acid: 7.5 ± 0.5 min, respectively stachyose 8.7 ± 0.2 min; raffinose 9.4 ± 0.2 min; sucrose 10.5 ± 0.2 min; Gal-Fru: 10.9 ± 0.2 min; glucose 11.5 ± 0.3 min; galactose 13.6 ± 0.2 min and fructose 15.5 ± 0.3 min.

The relative standard deviation of both systems is in the range of 3-10%.

2.21.3. Thin-layer chromatography: TLC

The aliquots from FTF, dextransucrase, invertase and α-glucosidase reactions were analysed as well using TLC. In order to obtain the best resolution for the mono- and disaccharides of the reaction mixture, the solvent system ethylacetate/ isopropanol/ water in a ratio of 6/ 3/ 1 (v/ v/ v) (at room temperature) was used as mobile phase.

The reaction samples were applied using “end to end pipettes” (3 or 5 µL volume from BLAURAND® intraEND, MERCK, Germany) on silica thin-layer plates (TLC aluminium

sheets 20 × 20 cm, silica gel 60 F254 with concentrating zone 20 × 2.5 cm – MERCK, Germany), at 1.5 cm under the concentrating zone after appropriate dilution (end concentration range between 1 and 5 g/L).

With regard to an optimal resolution the "multiple-ascents" technique was used. The saccharides were separated by using four ascents (4 × 90 min). Between the ascents, the plates were dried for 15 minutes at room temperature.

After the forth ascent the dried plates were developed by dipping into the detecting reagent using a CAMAG Chromatogram Immersion Device III (speed 2, time 4) (MERCK, Germany), followed by heating in an oven at 120°C for 15 minutes.

As developing reagent 0.3% (w/v) of N-(1-naphtyl)-ethylenediamine and 5% (v/v) concentrated sulphuric acid in methanol was used, due to its high sensitivity for most carbohydrates, which can be detected in the range of 50-2000 ng [52].

The sugars were visualised as dark spots on a pale pink background. The quantitative determination of the sugars was performed furthermore by using scanning densitometry. For this purpose a Bio-Rad Imaging Densitometer utilising Quantity One® Software (Version 4.2, Bio-Rad, Germany) was used.

The R_f were calculated taking as starting point the line where the samples were applied (at 1.5 cm under the concentration zone) and as end point the line of the mobile system front, up to where it migrated (between 14 and 16 cm).

2.21.4. Preparative cation-exchange chromatography

Stationary phase screening

Three types of stationary phases were tested for the preparative chromatographic separation of Gal-Fru: silicagel 60 (Fluka, Germany), Lewatit Monoplus M 500 in Cl^- form (Bayer AG, Germany) and PCR 6 in Na^+ form (Purolite International Ltd., France). The system ethyacetate/isopropanol/water in a ratio of 6/3/1 (v/v/v) was used as mobile phase for silicagel 60 and water for both two resins.

Gal-Fru separation

Separation of Gal-Fru from the reaction mixture was carried out with the microparticulate resin PCR 6, having an average beads size of 300-330 µm. PCR 6 is a strong acid cation exchange resin on basis of styrene-divinyl-benzene sulfonate with a 6% cross-linking

degree, monodispersed, having sodium as counter cation.

2100 mL hydrated resin was packed in a 2 m glass column (∅ = 3.9 cm) (Borosilicat 3.3, QVF, Germany), the resin bed having a length of 185 cm and being thermostated to a working temperature of 70°C with a Julabo V thermostat, Germany.

15 mL of immobilised dextransucrase/α-glucosidase or *S. oralis* dextransucrase reaction mixture with a total sugar concentration of maximum 400 g/L were subjected to the separation. The sugar mixture was loaded on the column and eluted with an average flow of 4 mL/min distilled water. The water was supplied with a Watson Marlow 5030 pump (Germany) and the eluted fractions were withdrawn with a Watson Marlow 101U pump (Germany), for both pumps being ensured a flow of circa 4 mL/min. The outlet of the column was coupled with a Knauer differential refractometer (Germany), which transmitted signals to a Kipp & EZ Zonen BD 41 recorder (Germany). After passing the detector, the eluted volume was collected and fractionated (at three minutes time intervals) using a Büchi 684 fraction collector (Büchi, Swiss).

After each separation, the resin was regenerated with a 3 M NaCl solution, having the two-fold volume of the packed resin. The regeneration was followed by a washing step, with distilled water, performed until the refractometer indicated a Bx° value of about 100.

2.21.5. Analysis for the Gal-Fru characterisation

All the analysis for the characterisation of the synthesised sucrose-analogue were performed with Gal-Fru having a purity of 92.5%.

NMR spectroscopy

For the NMR spectroscopic analysis 10 mg Gal-Fru was dissolved in 1 mL deuterium oxide (D_2O, 99.9% atom D, Aldrich Chemical Company Inc., USA).

Homonuclear and heteronuclear 1D- and 2D-NMR spectra were recorded on a Bruker AM-400 instrument (TU Braunschweig, Germany), operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C at 25 °C.

FTIR spectroscopy

FTIR spectra of 10 mg Gal-Fru, in pressed KBr discs, were recorded on a Bio-Rad FTS-25 spectrometer (Bio-Rad, Germany).

Melting point determination

Melting point was determined on a Melt-Temp II microscope (Laboratory devices, USA).

Optical rotation determination

Optical rotation values were measured with a Sucromat polarimeter (Dr. Kernchen, Germany), at 589 nm and 20°C.

Elementary analysis

Elementary analysis was carried out at Institute for Pharmaceutical Chemistry (TU Braunschweig, Germany) with an Elemental Analyser 1106 (Carlo Erba Instrumentazione).

Microscopy analysis

a. Microscopy

Freeze-dried Gal-Fru was analysed with a microscope Axioplus (Zeiss, Germany) with two microscope objectives of 20 x magnification and 10 x magnification (Leitz, Germany) having numerical apertures of 0.35 and 0.18, respectively. Detection unit: time-gated ICCD camera Picostar (LaVision Biotec, Germany). CCD-chip dimensions: 640 x 480 pixel. Evaluation software: DaVis 5.5.2 (LaVision Biotec, Germany).

b. Scanning electron microscopy

Gal-Fru freeze-dried particles were sprayed with gold for 85 seconds at 70 mA by using a Balzers SCD 040 (Germany). The preparations were then analysed with a transmission electron microscope (Philips EM 300, Germany) by applying an accelerating voltage of 20 kV. For photographing, black-white film negatives provided by Agfa Scientia (Germany) were used.

3. RESULTS

3.1. Galacturonic acid formation from extracted sugar-beet pulp

3.1.1. Exo-polygalacturonase screening

A number of commercial available enzyme preparations were screened for their ability to degrade pectin to galacturonic acid. Until now, some of these preparations such as those provided by Novo Nordisk, were designed especially for the use in food industry when a rapid depectinisation is required, for instance by the degradation of fruits and vegetables in the production of juices [34].

Many enzymes used in the food industry are crude, so it is not possible to measure the amount of enzymatically active protein responsible for substrate transformation (defined as specific activity). Therefore, the concentration of crude commercial enzymes is commonly expressed in units per mL solution. One unit (1 U) of exo-polygalacturonase (exo-PG) activity represents the enzyme mixture volume that catalyses the release of one µmol galacturonic acid (abbreviated as GA) per minute under assay conditions.

The results summarised in Tab. 3 reveal that from the variety of commercial enzyme products tested, in the reactions catalysed by Pectinex® 100L and Pectinex® ULTRA-SPL the highest activities and end concentrations were achieved. Since the lowest enzyme price per galacturonic acid concentration was calculated for Pectinex® 100L, it was concluded that this enzyme mixture is the most suitable for the hydrolysis of pectin. Accordingly, all the further investigations were performed with this enzyme preparation.

In the tests carried out with the mixtures Rohapect MPE and Rohament PL galacturonic acid was not detected. Rohapect MPE contains pectin esterases (enzymes that split ester bonds), but obviously no enzymes which are active on the α-(1-4)-linkages of the “smooth region” of pectin. Consequently, only the deesterification of pectin occurs, whereas the breaking down of the main chain to monomers can not take place. Quite the opposite is the Rohament PL preparation which contains just enzymes that are active towards “smooth region”. As a result, the pectin backbone is degraded to monomers which are esterified. Because of the lack of pectin esterases, the acetyl or methyl groups of the galacturonic acid esters formed can not be removed and subsequently, galacturonic acid can not be released.

Tab. 3: Exo-polygalacturonase screening

Enzyme preparation	Main activities	Activity [U/mL]	GA conc. after 7h [mmol/L]	Price/ end GA conc. [Euro/kg]
Gamapect press	Pectinase Pectin esterase Hemicellulase Cellulase	124	11	1.32
Gamapect plus	Pectinase Pectin esterase	232	27	0.48
Pectinex® 100L	Pectin transeliminase Polygalacturonase Pectin esterase	412	33	0.40
Pectinex® ULTRA-SPL	Pectinase Hemicellulase	289	34	0.46
Rohapect D5L Special	Pectinase Pectin esterase Pectin transeliminase	103	10	0.73
Rohapect MPE	Pectin esterase	-	-	-
Rohament PL	Pectinase	-	-	-

By the treatment of citrus pectin with Rohapect D5L Special, which is an enzyme preparation that consists of Rohapect MPE and Rohament PL supplemented with pectin transeliminase (or pectin lyase), galacturonic acid was detected. Despite the fact that Rohapect D5L Special showed the lowest values for exo-PG activity and galacturonic acid concentration, it can stand for an example for the combined activities of the two types of enzymes, respectively enzyme mixtures mentioned ahead.

After starting the reaction by adding Pectinex® 100L, galacturonic acid is released immediately, while oligomers are detected after longer reaction time intervals This behaviour may suggest that the enzyme preparation used encloses mainly exo-PG and possibly small amounts of endo-polygalacturonase (endo-PG). As described by the provider, Pectinex® 100L contains polygalacturonase, but information about the exact composition regarding the type of enzymes and their proportion is not offered.

The polygalacturonase enzymes catalyses the enzymatic cleavage of the O-glycosyl bond of α-(1-4)-polygalacturonan. The degradation pattern of the pectin chain proceeds either in a random or terminal fashion for endo-PG and respectively for exo-PG. Both types of enzyme require deesterified residues. Exo-PG attacks both “smooth” and “hairy” regions of pectin substrates by removing the galacturonic acid residues from the non-reducing end

of the pectin chain. As a result, galacturonic acid and oligomers with decreasing polimerisation degree are released [8]. It can be assumed that endo-PG, which is active towards the “smooth” homogalacturonic regions, might be also present and the oligomers formed may be the result of its random attack.

3.1.2. pH influence on Pectinex® 100L activity

The effect of pH on the exo-PG of Pectinex® 100L activity toward polygalacturonic acid (PGA) was examined in a pH range of from 3 to 5.4 at 50°C in sodium acetate-acetic acid buffer.

The optimal pH for PGA conversion to GA was found to be around 4 (maximum at 3.8) as shown in Fig. 7. Beyond and above this optimal range the enzyme activity decreases drastically, at pH values of 3 and 5 only no more than 50% activity could be detected.

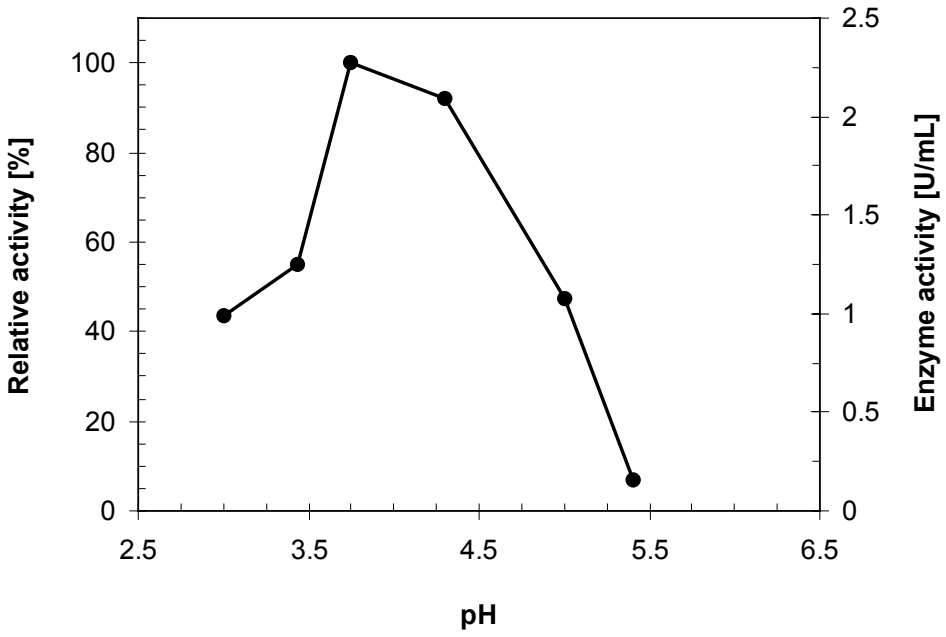


Fig. 7: pH influence on Pectinex® 100L activity with PGA as substrate (at 50°C in sodium acetate-acetic acid buffer)

This result does not confirm with the literature, where the optimal pH range of exo-PGs is reported to be much broader from 4 to 7. The discrepancy between the optimal pH values of the exo-PGs is caused by the different isolation sources of this enzyme (plant, fungi or bacteria) [11].

3.1.3. Effect of sodium and calcium cations on galacturonic acid production

It was previously demonstrated for exo-PG isolated from different sources, that Ca^{2+} increases the activity with approximately 20% [11]. In the experiments assayed by *Omran et al.* the influence of Na^{+} and Ca^{2+} cations on the exo-PG activity was examined [53]. In these investigations performed with exo-PGs isolated from different species of cucumbers, the maximal enzyme activity was achieved by adding 0.1 M NaCl and 0.4 mM $CaCl_2$ in the reaction medium.

Therefore, two experimental sets were carried out in this work in order to study the effect of these two cations on Pectinex®100L enzyme mixture. Both sets were performed by adding 0.1 M NaCl, 0.4 mM $CaCl_2$ and a mixture of both salts to 6 g/L SBP. The first series was carried out with 1 mL Pectinex® 100L (57.8 U) and the second with 2 mL Pectinex® 100L (115.6 U) at identical assay conditions.

In the first experimental set, the highest galacturonic acid production was attained in the tests without salt supplement. Moreover, it was noticed that contrarily to the literature, the enzymatic activity decreased in the reaction solutions enriched with salts in following supplementing order: 0.4 mM $CaCl_2$ (94.7% relative activity), 0.1 M NaCl (86.5% relative activity) and salts mixture of 0.4 mM $CaCl_2$ and 0.1 M NaCl (85% relative activity) as shown in Fig. 8.

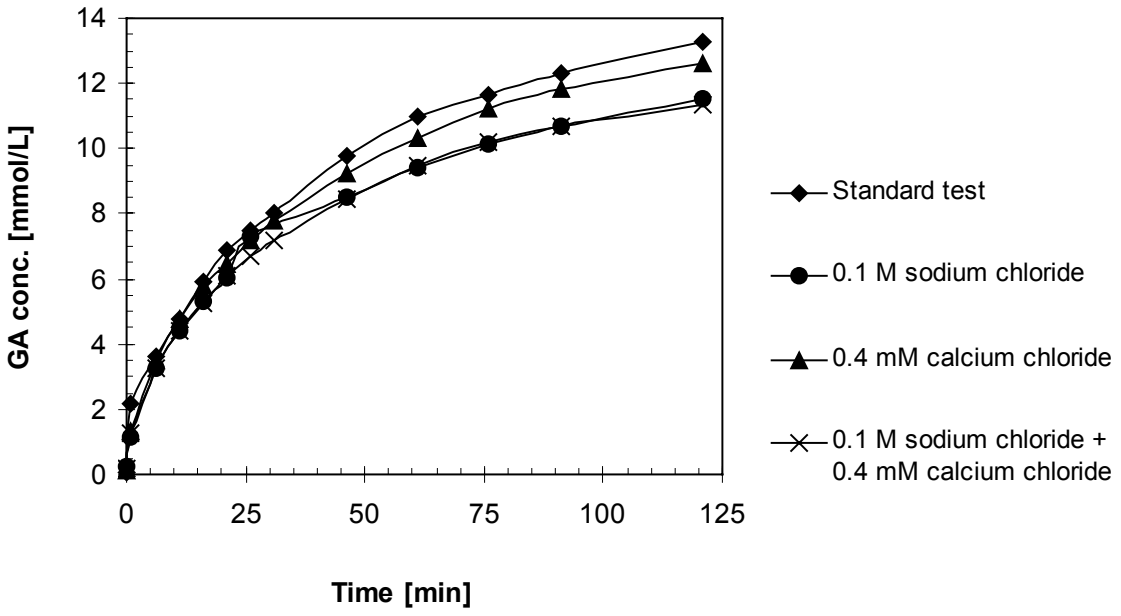


Fig. 8: Influence of NaCl und $CaCl_2$ on the galacturonic acid formation (6 g/L SBP and 1 mL (57.8 U) Pectinex® 100L in sodium acetate-acetic acid buffer pH 4 at 50°C)

The doubling of the enzyme volume to 200 µL per 100 mL reaction solution, in the second reaction series, led to results in accordance to those achieved in the first experimental set. As Fig. 9 shows, an increase of the galacturonic acid production was attained neither by distinct single salt adding (NaCl or $CaCl_2$) nor by both salts mixture enrichment (NaCl and $CaCl_2$) of the reaction medium. The maximal activity loss was 13.2% in the tests with 0.1 M NaCl and with the two salt mixture as summarised in Tab. 4 and Tab. 5 (in these tables the initial reaction rates and the calculated yields are also summarised).

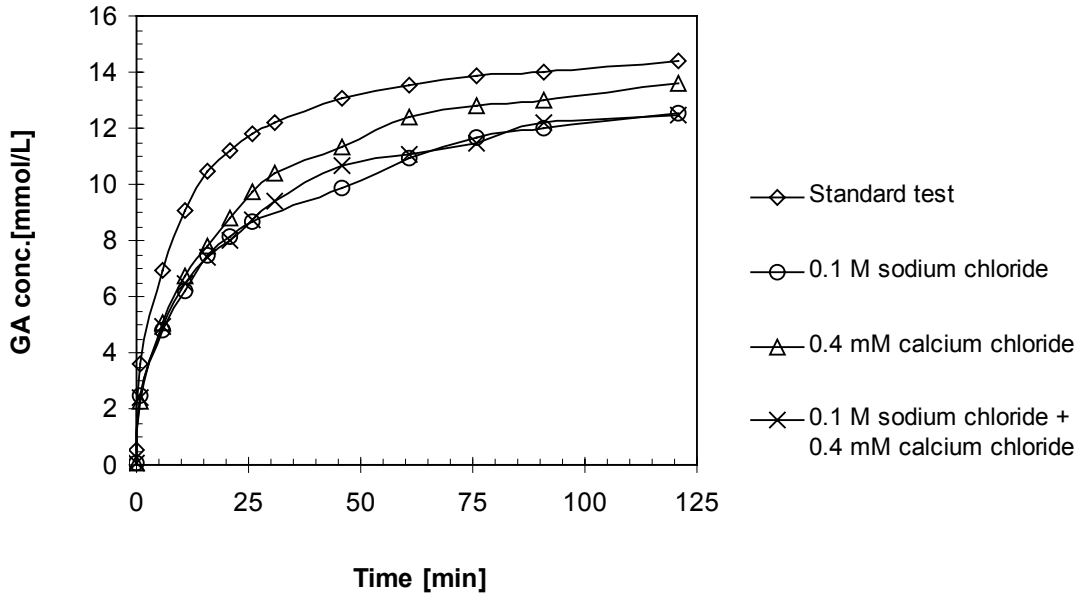


Fig. 9: Influence of NaCl und $CaCl_2$ on the galacturonic acid formation (6 g/L SBP and 2 mL (115.6 U) Pectinex® 100L in sodium acetate-acetic acid buffer pH 4 at 50°C)

Contrary to the expectations, it is evident that by sodium and calcium salt addition to the reaction medium no influence on the exo-PG activity was manifested. The activity loss of about 15% can be disregarded, since it may be owed to experimental errors.

An increase of the exo-PG activity from most sources is determined by a concentration up to 0.5 mM Ca^{2+} and monovalent cations, especially Na^{+}, are reported to be inhibitory for some exo-PGs [8]. Therefore, several reasons could be taken into consideration for explaining these results. Firstly, the source of the enzyme plays a very important role explaining the dissimilar behaviour of different exo-PGs: the exo-PG described in the literature was proceeded from vegetative tissues (different types of cucumbers during ripening) and the exo-PG contained in the enzyme preparation Pectinex®100L was

produced from bacteria, by a selected strain of *Aspergillus niger*.

Tab. 4: First set results (100 µL Pectinex® 100L per 100 mL reaction solution)

Parameter	6 g/L SBP + 57.8 U Pectinex® 100L, pH 4, 50°C			
	Standard test	0.1 M NaCl	0.4 mM $CaCl_2$	0.1 M NaCl + 0.4 mM $CaCl_2$
GA conc. [mmol/L]	13.3	11.5	12.6	11.3
Relative activity [%]	100.0	86.5	94.7	85.0
Yield [%] (after 120 min.)	61.3	51.0	58.0	52.0

Tab. 5: Second set results (200 µL Pectinex® 100L per 100 mL reaction solution)

Parameter	6 g/L SBP + 115.6 U Pectinex® 100L, pH 4, 50°C			
	Standard test	0.1 M NaCl	0.4 mM $CaCl_2$	0.1 M NaCl + 0.4 mM $CaCl_2$
GA conc. [mmol/L]	14.4	12.5	13.6	12.5
Relative activity [%]	100.0	86.8	94.4	86.8
Yield [%] (after 120 min.)	67.0	58.0	63.0	58.0

Secondly, the cations could have an effect not necessarily directly on the exo-PG, but also on the other present enzymes in Pectinex®100L, especially on the endo-PG. This influence could indirectly affect the exo-PG by the other enzymes and by this consequently the galacturonic acid production.

Thirdly, as *Walter et al.* previously reported the calcium cations can interact with the polygalacturonans to form aggregates, which are considered to be unavailable for the exo-PG attack [8].

Besides, endo-PG is contained in small amounts in the used enzymatic preparation. This is a major extracellular enzyme secreted by bacteria and fungi and in contrast to exo-PG is produced in these microorganisms in much more quantity. Its presence could give explanation to the non-requirement of monovalent cation and to the inhibitory effect of Ca^{2+} at concentration as low as 0.4 mM.

3.1.4. Pectin extraction from sugar-beet pulp

With regard to an application both on lab- and technical scale, prior to enzymatic treatment the cell-wall polysaccharides, respectively the pectin from sugar-beet pulp has firstly to be solubilised. Otherwise, the formation of galacturonic acid can not occur (or extremely low) since the cell-wall is resistant to the direct attack of exo-PG. Commercial pectin extraction occurs by means of chemical/physical methods at pH 1.5-3.0 and 60-100°C. The extract may be isolated by precipitation as pectin salts or with ethanol or isopropanol. The isolate may be concentrated afterwards by different methods to result in liquid or solid form [8]. High pressure and temperature, mild and aggressive reagents can ensure the digestion of the cell-wall enabling the extraction of pectin by solubilisation. The resulting mixture consisting of pectin extract and pulp residues can be used as substrate and may be subjected to the treatment with Pectinex® 100L, as described below. On this pathway, the galacturonic acid can be released without difficulty, the yield can be calculated and consequently, each pectin extraction test can be evaluated.

The basis for the pectin extraction series was the patented method developed by *Herbstreith and Fox* [33]. By using this method, the pectin extraction occurs in citric acid solution at a pH of 3.0, for six hours at 95°C. Due to it's suitable properties, the sugar-beet pectin extract obtained with on this pathway is utilised industrially for biscuits, ice-cream and mousse production.

Tab. 6 summarises the results obtained by extraction of pectin with different reagents at several temperatures. As reported in the literature, the galacturonic acid content of dried extracted sugar-beet pulp is in the range of 8-16% [6].

The most efficient extraction was achieved with sulphuric and citric acid at elevated temperatures, 130°C and 145°C under pressure (by autoclaving). The highest galacturonic acid yield was achieved already after 30 minutes, when the solubilisation of pectin occured at 130°C in the presence of sulphuric acid (test nr. 9). Comparable results were accomplished by the treatment with citric acid also at 130°C (test nr. 7).

The extraction of pectin by treatment with the basic reagent, sodium hydroxide at 130°C (test nr. 10) led only to an average value for galacturonic acid yield. For all acid and basic reagents, by increasing the temperature to 145° and thus the pressure, no further enhancement regarding the galacturonic acid yield was achieved.

Tab. 6: Pectin extraction results

Test nr.	Reagent	Time [h]	Temperature [°C]	pH	Galacturonic acid [% dry weight]
1	Water	1.5	95	6.5	6.6
2	Water	6.5	95	6.5	5.6
3	Formic acid	1.5	95	3.0	6.8
4	Citric acid	1.5	95	3.0	3.7
5	Citric acid	3.5	95	3.0	5.9
6	Citric acid	6.5	95	3.0	9.6
7	Citric acid	0.5	130	3.0	12.4
8	Acetic acid	0.5	130	3.0	10.9
9	Sulphuric acid	0.5	130	3.0	12.8
10	Sodium hydroxide	0.5	130	10.0	9.7
11	Citric acid	0.5	145	3.0	12.2
12	Acetic acid	0.5	145	3.0	11.0
13	Sulphuric acid	0.5	145	3.0	12.4
14	Sodium hydroxide	0.5	145	10.0	9.8

It can be concluded that at temperatures much higher than 95°C and lower pH values (pH 3) the extraction of pectin is evidently favoured. However, with respect to economical constraints in order to minimise the reagents and energy costs, the solubilisation at 95°C (so without pressure) and free of any type of reagent was chosen as appropriate method (test nr. 1: despite the fact that the galacturonic yield represent only the half of the maximal value achieved by the treatment with sulphuric acid at 130°C under pressure).

It has to be mentioned that in view to optimise the galacturonic yield, the sugar-beet pulp was additionally subjected before and after the pectin extraction to several mechanical treatments. The sugar-beet pulp was smashed in a food-mixer and the resultant puree/mash was then used for pectin extraction with water at 95°C. Separation of the pulp by pressing and filtering methods followed the extraction procedure. All the additional mechanical treatments tested directed to no significant increase of product yield. Accordingly, for the kinetic series with sugar-beet pulp the pectin extraction was performed without any further mechanical treatment of this renewable material.

3.1.5. Kinetics of galacturonic acid release

3.1.5.1. Determination of kinetic parameters

In order to characterise the enzyme mixture with regard to the galacturonic acid release and to model the catalysis for a bioreactor system, the apparent kinetic constants had to be calculated. For this purpose the release of galacturonic acid was studied by initial rate measurement under steady-state conditions (for the first 20 minutes of the reaction). It has to be emphasized that the kinetic parameters were determined from the galacturonic acid formation and not from the substrate consumption experimental data. The fundamental reason for calculating kinetics from the product release is the fact that for pectin, as for every polymer, it is not possible to attribute one distinct molecular weight since they are polydisperse molecules characterised by an average molecular weight. Besides, polymers, respectively pectin are not detectable quantitatively with the analytical tools used in this work (HPAEC and IC). Therefore, the calculation of the kinetic parameters based on substrate degradation experimental data can not be achieved.

Having the initial rates r, the r_{max} and "apparent" K'_M values were estimated using three classical linearisation methods Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee (mentioned in section 2.10).

The term "apparent" for the parameters (symbolised by the apostrophe, for example K'_M instead of K_M) was introduced with consideration to the fact that the kinetics will be described for an enzyme mix and not for a pure catalyst. The enzyme of interest in Pectinex® 100L, responsible for the galacturonic acid formation is the exo-PG, but other enzymes present in the preparation may also show side activities influencing the kinetics of the galacturonic acid release.

Extracted sugar-beet pulp (ESB) represents a substrate with a varying complex, non-standardisable composition (determined by weather conditions and soil texture). This could cause difficulties for the determination of kinetic parameters. For this reason, the first kinetic series were performed with citrus- and sugar-beet pectin as reference substrates (sections 3.1.5.2, 3.1.5.3 and 3.1.5.4), because of their better defined composition. These tests should permit the calculation of the apparent kinetic constants for the much more complex substrate extracted sugar-beet pulp. These constants could be taken as first attempt for the modelling of the enzyme action towards sugar-beet pulp.

3.1.5.2. Substrate: citrus pectin (CP)

On the basis of the experimental data obtained for galacturonic acid release from citrus pectin in the concentration range from 1 g/L to 25 g/L, the initial reaction rates *r* were calculated. The initial reaction rate rises for substrate concentrations up to 25 g/L. At higher concentration the strongly increasing viscosity due to the accumulation of high-molecular-weight oligomers has to be taken into account, since it hinders a rapid mixture between the enzyme and reaction solution. For this reason, the test with 50 g/L substrate was disregarded for the mathematical evaluation.

The resulted *r* values were used for the determination of the kinetic constants by the three mentioned linearisation methods (section 2.10). The calculated values for r_{max} and K'_M for citrus pectin with the three classic linearisation methods are summarised in Tab. 7.

Tab. 7: Experimental apparent kinetic parameters for citrus pectin (100 µL Pectinex® 100L per mL reaction solution at pH 4 in sodium acetate-acetic acid buffer and 50°C)

Parameter	Lineweaver-Burk	Eadie- Hofstee	Hanes-Woolf
$r_{max\ CP}$ [mmol GA/(L × min)]	0.93	1.08	1.03
$r_{max\ CP}$ [g GA/(L × min)]	0.18	0.21	0.20
$K'_{M\ CP}$ [g CP/L]	10.00	11.90	11.80

The sets of experimental results obtained from each method are in a good agreement, showing a variation of maximal 16% for the $K'_{M\ CP}$ values. These variations between the results could not only be attributed to the experimental errors, but also to the different ranges of statistical margin of error of each linearisation method.

Based on the experimental data for the kinetics of the galacturonic acid release from each pectin type substrate an equation was developed and computed. Starting with a Michaelis-Menten-equation, the differential equation was modified with the incoming experimental data. It includes terms of competitive inhibition by the product (galacturonic acid) K'_{IP} and substrate excess inhibition K'_{IS} as presented below:

$$r = \frac{r_{\max} \cdot [S]}{K'_M \cdot \left(1 + \frac{[P]}{K'_{IP}}\right) + [S] \cdot \left(1 + \frac{[S]}{K'_{IS}}\right)} \quad (4)$$

where the terms *[S]* and *[P]* of the equation represent the substrate and respectively product concentration.

Without any exception, in all the figures presented in the “Kinetics” section 3.1.5, the curves correspond to the values modelled with the developed equation and the points to the experimental results.

The experimental results of the release of galacturonic acid from different citrus pectin start concentrations and the curves modelled by using equation 4, are presented in Fig. 10 and Fig. 11.

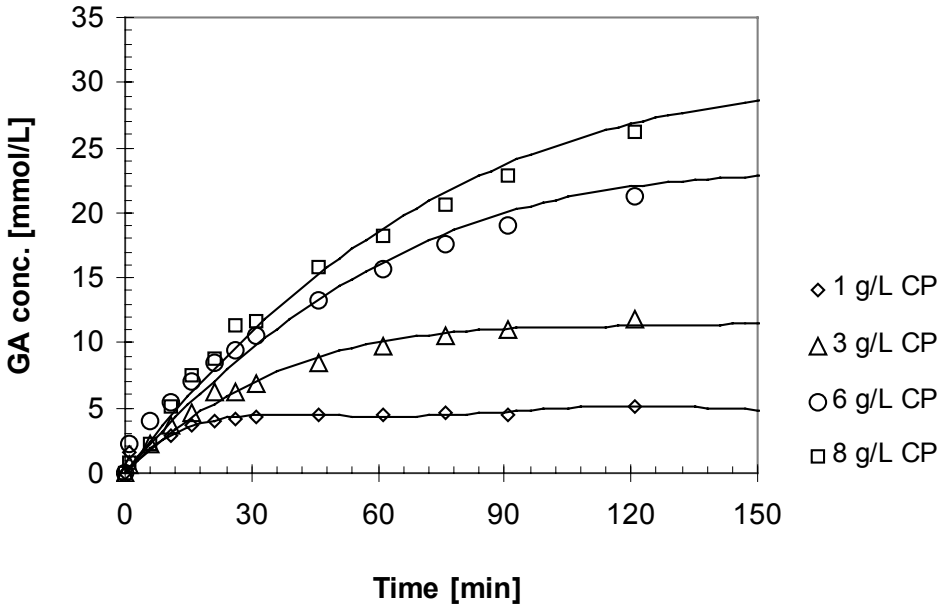


Fig. 10: Citrus pectin conversion to galacturonic acid (low substrate concentrations)

As it can be seen, for low substrate concentrations, the calculated and modelled values correspond very closely to the experimental data (Fig. 10). At higher substrate concentrations minor deviations, which could not be explained because of their non-systematic occurence, are given (Fig. 11).

By iterative parameter optimisation performed with the program “Modelmaker 3.03” (section 2.10) using the developed equation, the polygalacturonic acid content of citrus pectin was determined to be approximately 74%, a result which agrees to literature data (section 2.1) [6].

Variation of the pH value was observed despite the buffered medium. In the case of 3 g/L citrus pectin, the pH shifted within 180 minutes from 4 to 3.7. For higher substrate concentrations 25 and 50 g/L, the pH decreased from 4 to 3.3 and respectively to 3.0.

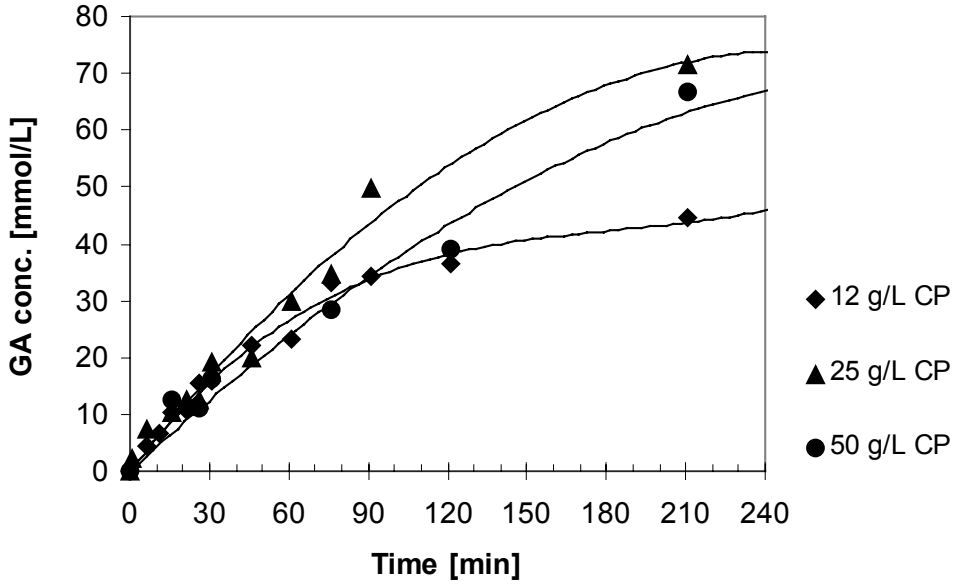


Fig. 11: Citrus pectin conversion to galacturonic acid (high substrate concentrations)

This variation of pH is related to the synthesis of galacturonic acid and most probably to the action of esterases, represented by pectin acetylesterases, which are liberating acetic acid in the medium. Consequently, the pH decrease is the direct result of the accumulation of both acids in the reaction solution.

3.1.5.3. Product inhibition

On account of the fact, that in a technical process, from high substrate concentrations high product amounts are produced, the influence of the galacturonic acid on the reaction rate was studied. From several experiments over a longer period it seemed possible that the reaction product, galacturonic acid, may inhibit the enzyme activity. Therefore, two experimental pathways were followed (see section 2.9).

The first test series was performed with increasing galacturonic acid concentrations in the reaction medium (Fig. 12) and the second experimental row with increasing substrate concentrations at constant galacturonic acid concentrations (Fig. 13).

Without addition of the product, the catalysis of the reaction runs with the highest rate, with increasing product start concentrations the rate decreases.

Variations of the pH were also in this test series notified. The maximal pH decrease was detected for the test with 8 g/L citrus pectin and 12 g/L (0.062 mol/L) galacturonic acid, a

decrease from 4 to 3.2 (within 180 minutes).

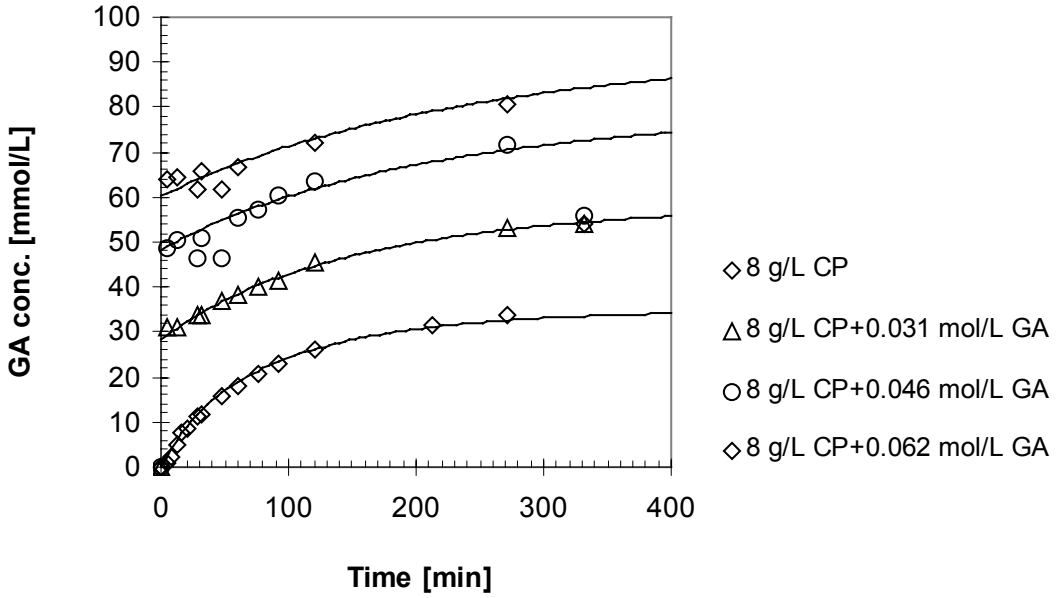


Fig. 12: Influence of increasing galacturonic acid concentrations on its formation

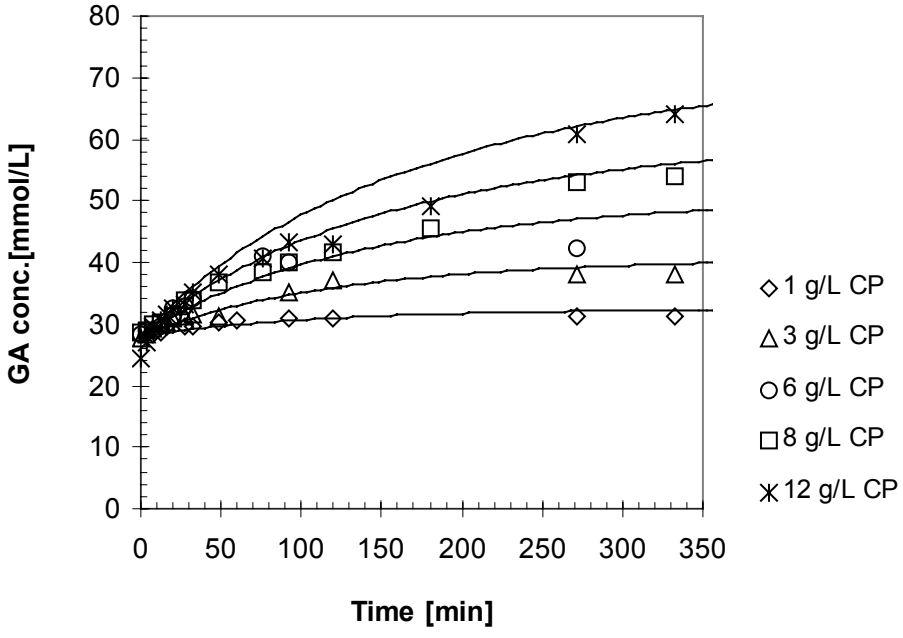


Fig. 13: Influence of galacturonic acid on its formation at varying citrus pectin concentrations

As Fig. 13 shows, the inhibition effect can be reduced, if the substrate concentration is increased, which is usually related to a competitive type of inhibition.

The apparent substrate affinity constant $K'_{M\ CP}$, calculated with the mathematical model, for citrus pectin is 3.5 g/L. The product inhibition is significant, for K'_{IP} a value of 5.4 mmol/L could be calculated. The substrate inhibition parameter K'_{IS} was found to be 290 g/L, a value that is not relevant for a substrate inhibition, for the fact that it lies out of the solubilisation capacity of citrus pectin (which is approximately 50 g/L).

With these parameters the influences of substrate and product concentration on the reaction rate can be described in a three dimensional diagram as illustrated in Fig. 14.

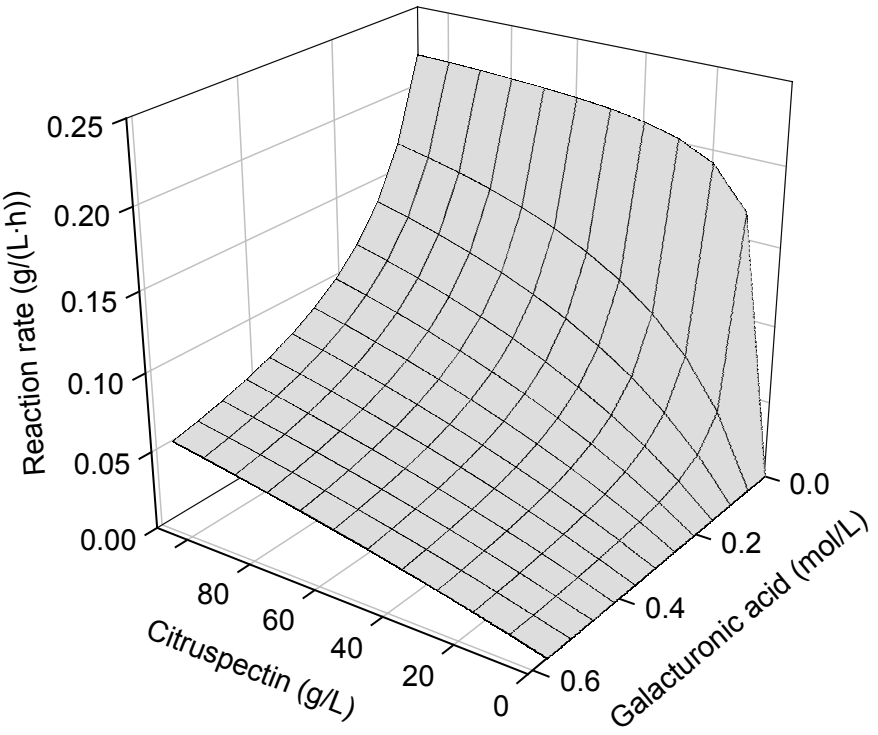


Fig. 14: Influence of substrate and product on the enzyme mixture activity

Already for low product concentrations a significant reduction in the reaction rate take place. For example at 0.05 mol/L and 0.6 mol/L galacturonic acid at a citrus-pectin concentration of 60 g/L, a 30% and respectively a 80% loss of activity can be calculated.

3.1.5.4. Substrate: sugar-beet pectin (SBP)

For sugar-beet pectin, the apparent kinetical constants calculated with the experimental data with the three classic linearisation methods (summarised in Tab. 8) are in a good agreement (the $r_{max\ SBP}$ values match in fact entirely). Between the determined values for the sugar-beet pectin affinity constant $K'_{M\ SBP}$, a dissimilarity of circa 17% is given (3.6 g/L obtained with Lineweaver-Burk and 3.0 g/L for Eadie-Hofstee linearisation).

Tab. 8: Experimental apparent kinetic parameters for the substrate sugar-beet pectin (100 µL Pectinex® 100L per mL reaction solution at pH 4 and 50°C in sodium acetate-acetic acid buffer)

Parameter	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf
$r_{max\ SBP}$ [mmol GA/(L × min)]	0.36	0.36	0.36
$r_{max\ SBP}$ [g GA/(L × min)]	0.07	0.07	0.07
$K'_{M\ SBP}$ [g SBP/L]	3.6	3.0	3.5

In the case of sugar-beet pectin, the initial reaction rate measurements indicate the same tendency as for citrus pectin. From experimental data and modelled curves, shown in Fig. 15 it was calculated that with increasing substrate concentration the rate increases, whereas the yield decreases.

The numerical integration with the Runge-Kutta method gave a 41% galacturonic acid content, that corresponds to circa 13% deviation from the literature values of 54-78%. One aspect which could explain this result may be the varying composition of this natural product, due to geological and weather conditions.

With the same calculation method, for the substrate inhibition constant K'_{IS} a value a of approx. 300 g/L was determined, which as well as in the case of citrus pectin can be disregarded owing to the fact that the solubilisation limit for sugar-beet pectin lies at a concentration of about 30 g/L and so, the obtained value is out of the studied concentration range.

The computed value for the substrate affinity constant $K'_{M\ SBP}$ is 3.0 g/L and for the product inhibition constant K'_{IP} is 0.8 mmol/L. This reveals that the product concentration seems to have a much stronger inhibiting effect than in the case of citrus pectin as substrate.

It has to be mentioned that special emphasis concerning product inhibition laid on these kinetics studies during the tests only with citrus pectin.

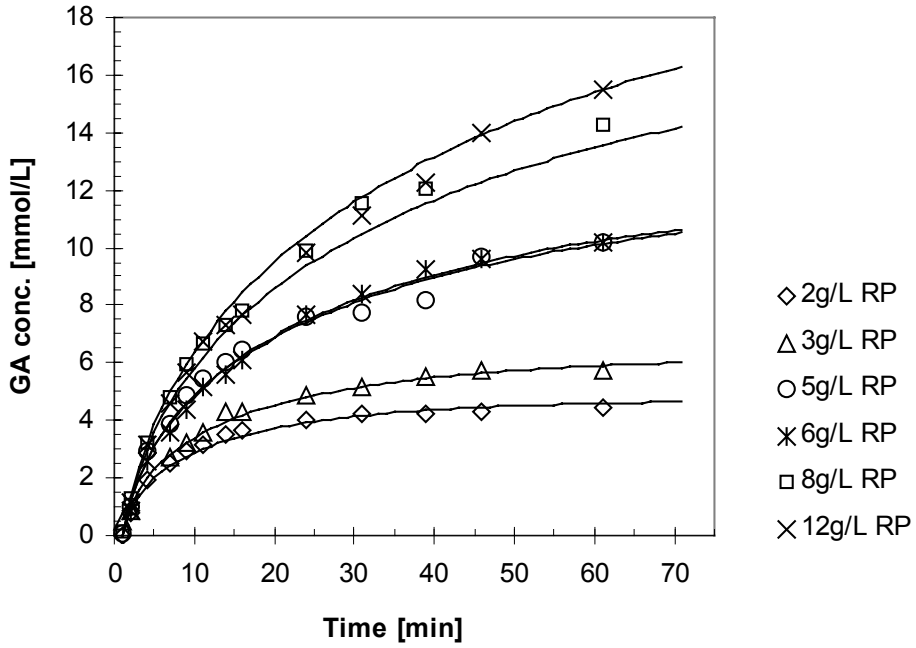


Fig. 15: Substrate kinetics of the sugar-beet pectin degradation

After 180 minutes, the pH shift was of 0.5 pH units for the highest sugar-beet pectin concentration (12 g/L) and therefore of minor effect concerning the reaction rate.

3.1.5.5. Substrate: extracted sugar-beet pulp (ESB)

After the experiments with relatively good defined substrates, kinetic tests with extracted sugar-beet pulp were performed. With respect to industrial use, these studies were carried out in a non-buffered system. Due to the source of sugar beet pulp (sugar-beet extraction), relatively high Ca^{2+} concentrations are given, ensuring a relative stable pH. The pH of the reaction medium was 4.4, matching to the optimal enzyme activity range.

As shown in Fig. 16, the initial reaction rate rises up to a concentration of 200 g/L substrate (60 g/L dry substance). At higher sugar-beet pulp concentrations (250 g/L and 300 g/L equivalent to 75 g/L and 90 g/L dry substance respectively) the reaction rate decreases.

The reason for this is given by experimental problems, which appeared for sugar-beet pulp concentrations higher than 150 g/L caused by the high volume of the pulp. It is well-known that dried sugar-beet pulp swells tremendously in water of about four-five fold. This issue led to an incomplete covering of the substrate with the reaction medium, to a partial

mixing of the enzyme with the reaction solution and subsequently, to a relatively small contact surface for the enzymatic attack. This deficiency could be overcome on large-scale by recirculation of the enzyme solution. On this basis, the data obtained from the experiments with sugar-beet pulp concentrations higher than 150 g/L were not taken into account regarding the calculation of the kinetic constants.

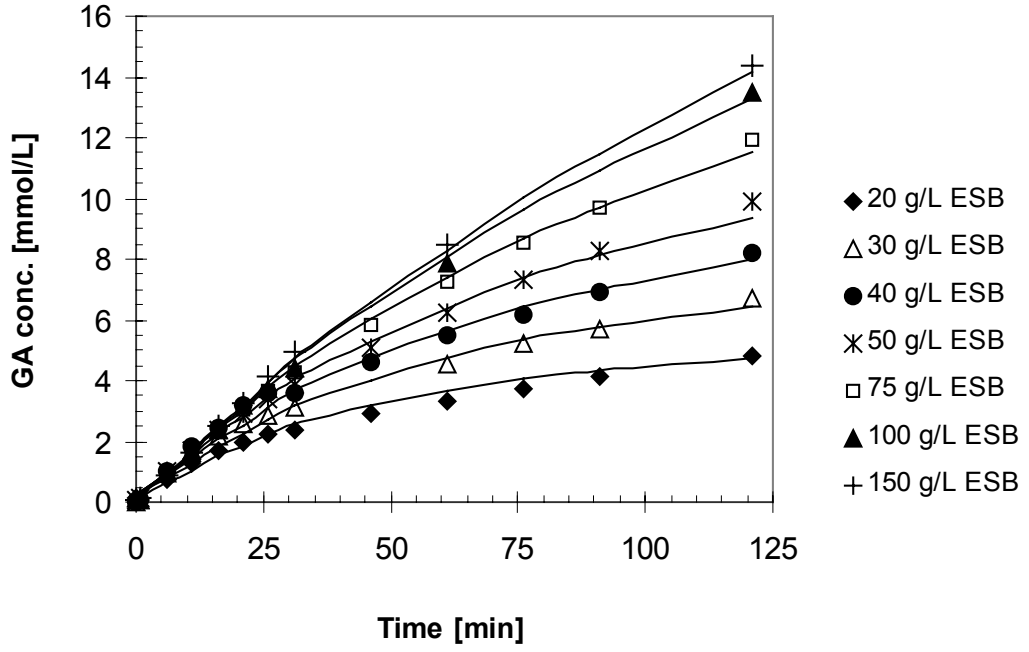


Fig. 16: Substrate kinetics with freeze-dried, pressed, extracted sugar-beet pulp

The mathematical calculation gave a 4.8% galacturonic acid turn-over from sugar-beet pulp, corresponding to 16% dry substance, a value which lies in the field of the literature data (8-16%).

The substrate inhibition constant K'_{IS} was found to have a value of 350 g/L, which as well as in the case of both reference substrates, citrus- and sugar-beet pectin, can be disregarded. The value of the substrate affinity constant $K'_{M\ ESB}$ was calculated to be 25 g/L and for the product inhibition constant K'_{IP} 2.6 mmol/L.

After 180 minutes, the degradation of extracted sugar-beet pulp in water led to a pH decrease of one pH unit from 4.4 to 3.4, a value that agrees to those obtained in the tests series with citrus- and sugar-beet pectin.

3.1.6. Separation and concentrating of galacturonic acid

Pectinex®100L hydrolyses pectin substrates to galacturonic acid, arabinose and galactose and oligomeric compounds (here not studied). For that reason, in order to isolate the product from the other saccharidic compounds on lab-scale, preparative cation exchange chromatographic separation was tested.

The cation exchange resin Finex CS 13 GC, which is by now used technically in water softening, demineralisation and condensate purification, was selected as stationary phase.

The separation was investigated at four pH values 2.2, 3.2, 4.2 and 5.2 as the chromatograms from Fig. 17-Fig. 20 illustrate. As it can be seen, the first compound that emerges is the galacturonic acid (after approx. 200 mL elution volume), followed by the neutral monosaccharides galactose (after approx. 300 mL elution volume) and arabinose (after approx. 400 mL elution volume). The results show that a relatively good separation of galacturonic acid could be achieved at pH 4.2.

After each separation step, by combining the purest eluted fractions, from approx. 200 mL to 300 mL eluent volume, products with purities higher than 70% were obtained.

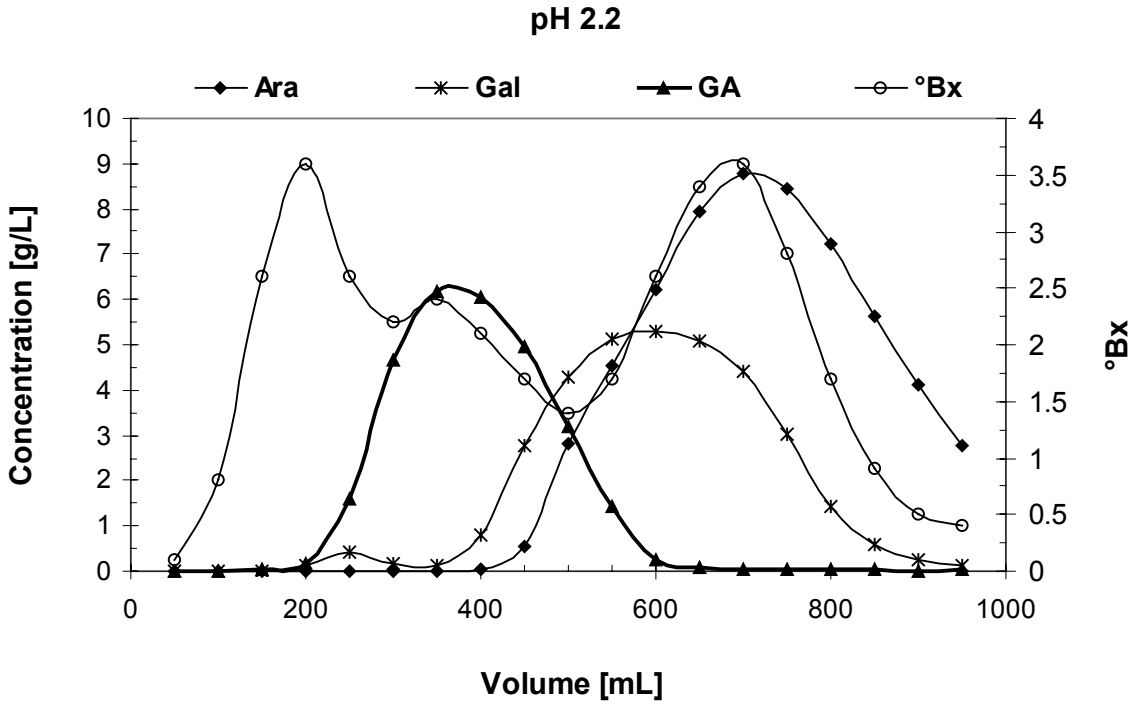


Fig. 17: Galacturonic acid separation at pH 2.2

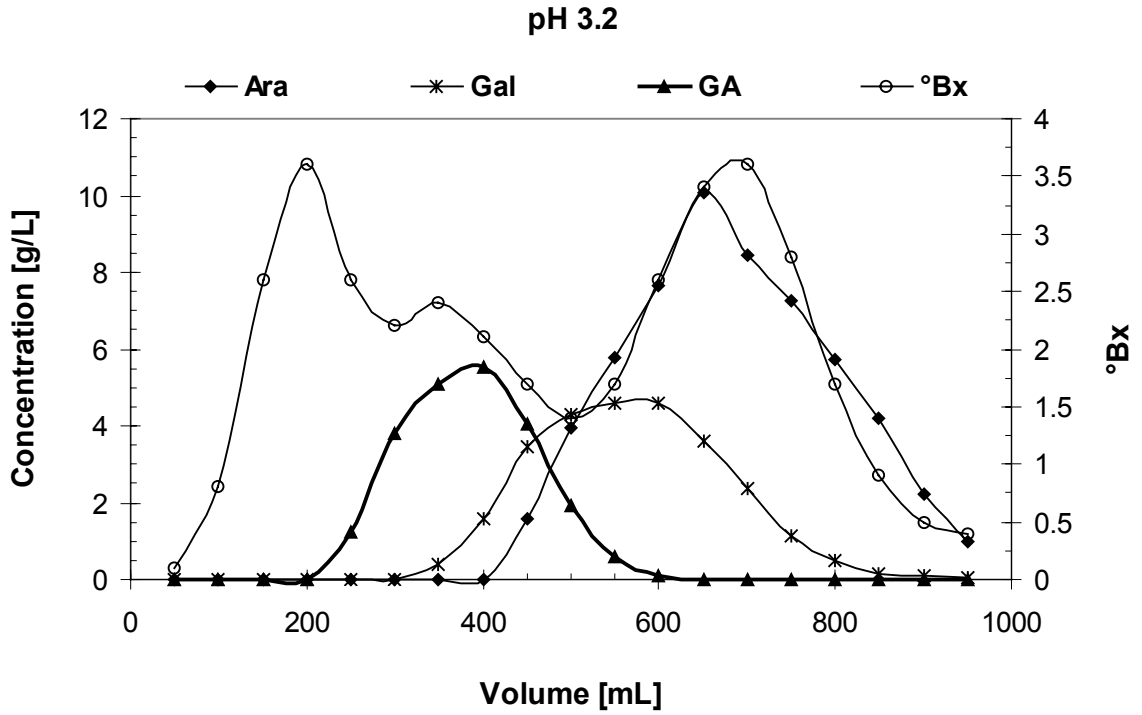


Fig. 18: Galacturonic acid separation at pH 3.2

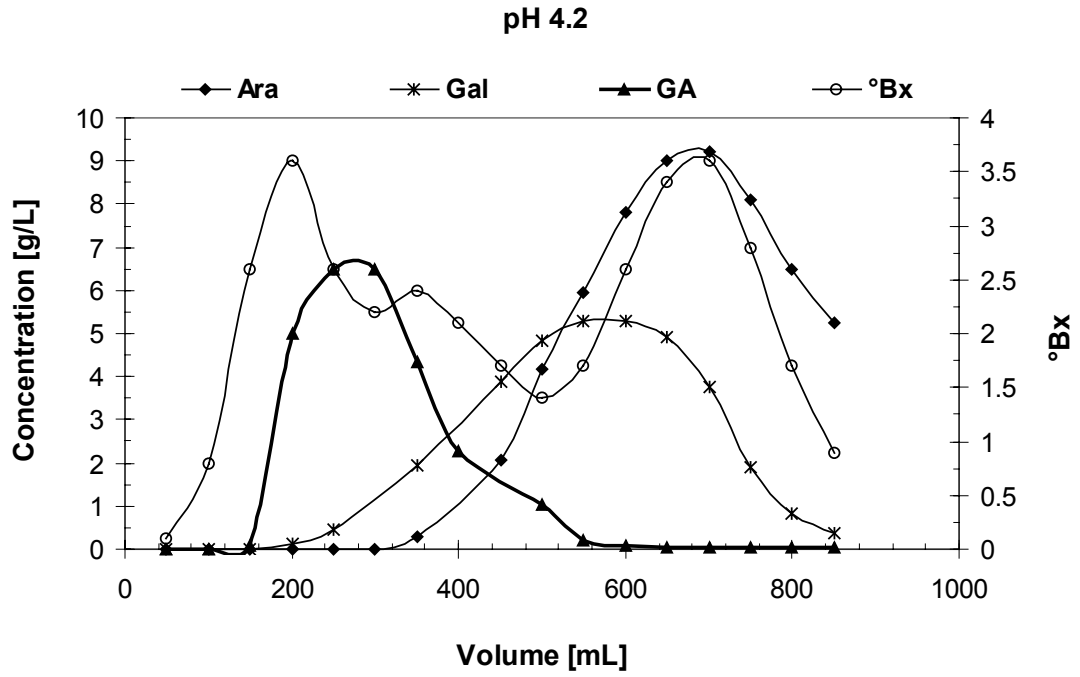


Fig. 19: Galacturonic acid separation at pH 4.2

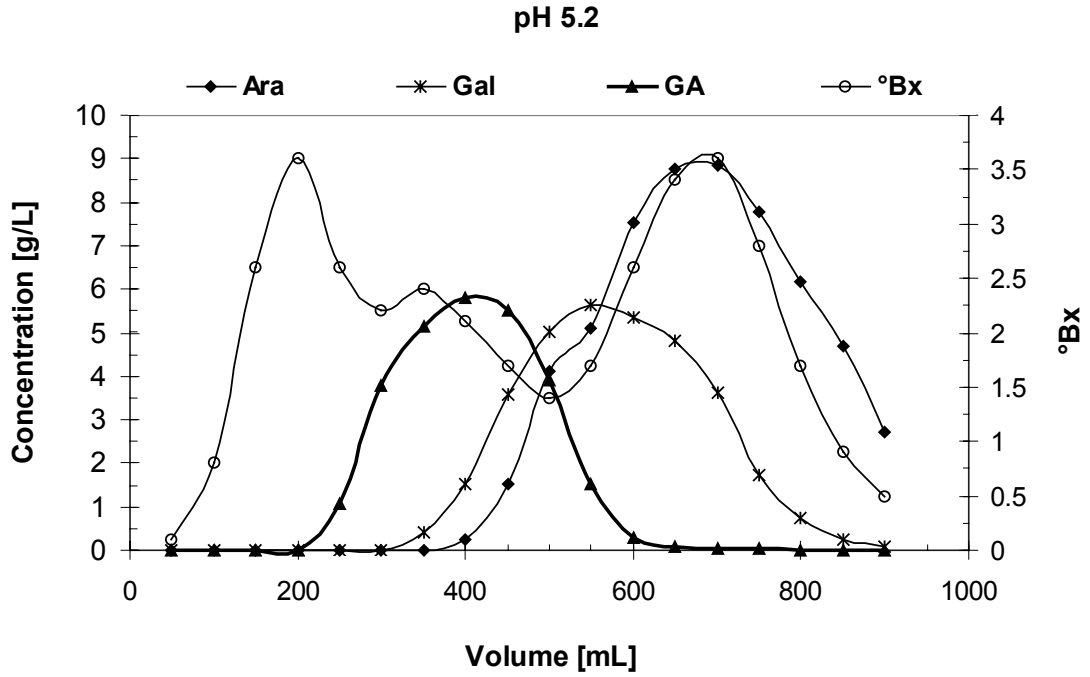


Fig. 20: Galacturonic acid separation at pH 5.2

The refractometric dry substance analysis (°Bx values) indicated furthermore that the removal of the inorganic salts could be not attained by separation with this type of cation exchange resin.

The disadvantage of this method is that the galacturonic acid is diluted by elution, a fact that imposes a supplementary concentrating step. With regard to a feasible technological application, galacturonic acid should be not simply purified but also made available, if not in solid form at least in a concentrated form. This purpose can be achieved by using two effective concentrating methods: adsorption onto ion exchange resins or evaporation.

With respect to the process costs, for the fact that the evaporation involves exceedingly high energy consumption, the concentrating was performed by adsorption. The strong basic anionic exchange material Lewatit Monoplus M 500 in acetate form was selected as stationary phase, the sample being eluted with phosphate buffer.

After the first desorption test, the capacity of Lewatit Monoplus M 500 for galacturonic acid was calculated to be of 26 mg GA/g resin. For repeated use of the resin, experimental data revealed that the cation exchange resin capacity increases about four-fold from 26 to 98 mg GA/g resin. Accordingly, it can be supposed that the conditioning with acetate, may

succeed firstly by elution with phosphate buffer. On the other hand, the repeating of the conditioning step may ensure the complete equilibration of the resin with acetate.

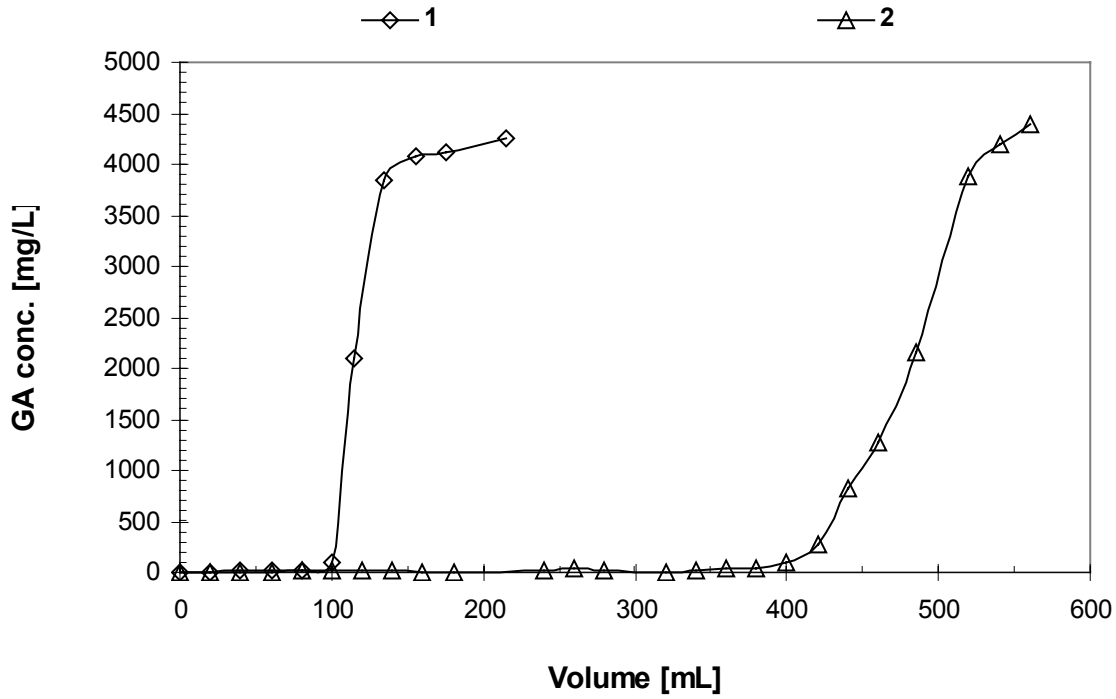


Fig. 21: Capacity of Lewatit Monoplus M 500 for galacturonic acid by repeated use: 1-first cycle and 2-second cycle

Therefore, in order to examine the influence of the conditioning on the packed resin three test series were conducted. For all series, a 20 g Lewatit Monoplus M 500 sample was stored for 48 hours in the appropriate conditioning solution. The conditioning solution was for the first series 1 M acetic acid, for the second phosphate buffer (pH 8.1) and for the third, 2 M sodium hydroxide.

The results illustrated in Fig. 22 revealed that for all the conditioning reagents, a considerable increase of the resin adsorption capacity for galacturonic acid was attained. The 48 h conditioning in acetic acid and phosphate buffer led to similar capacity values of approx. 220 mg GA/g resin, whereas with sodium hydroxide 325 mg GA/g resin were achieved. The increase of capacity was calculated to be more than 8-fold for phosphate buffer and acetic acid and of 12.5-fold for sodium hydroxide when compared to the first concentrating test discussed above (26 mg GA/g resin).

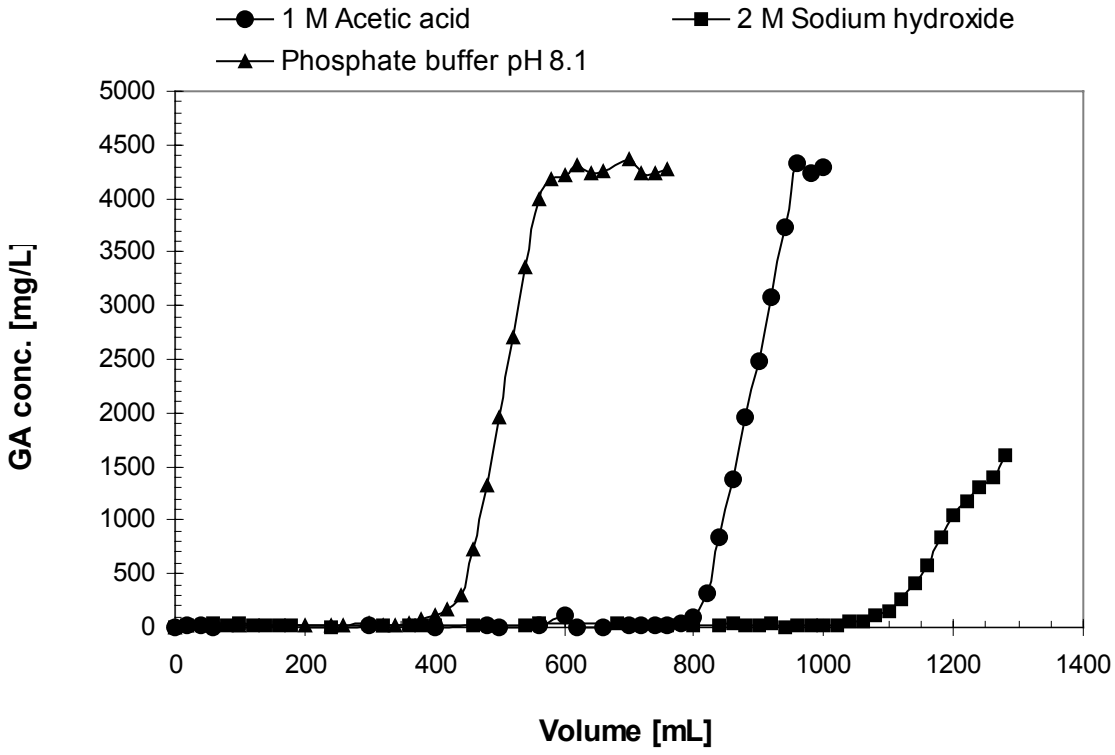


Fig. 22: Capacity of Lewatit Monoplus M 500 for galacturonic acid after conditioning for 48 h with: 1-acetic acid; 2-phosphate buffer and 3-sodium hydroxide

The influence of pH on the elution of galacturonic acid was also investigated. The results illustrated in Fig. 23 show that at a pH value of 8.1 the adsorption achieves a value nearly twice so high (900 mg GA/L) compared to the one obtained for pH 9.1 (575 mg GA/L). According to these results, the concentrating of galacturonic acid is favoured at pH 8.1.

A combination of sodium hydroxide conditioning of Lewatit Monoplus M 500, followed by elution at pH 8.1 led to a further concentrating of galacturonic acid of more than 20 g/L.

Based on these results it can be concluded that galacturonic acid can be well separated from the other saccharidic compounds by cation exchange chromatography with the resin Finex CS 13 GC. The subsequent concentrating step by adsorption onto the strong anion exchange material Lewatit Monoplus M500 was successful leading to a significant concentrating of the product of interest, the galacturonic acid.

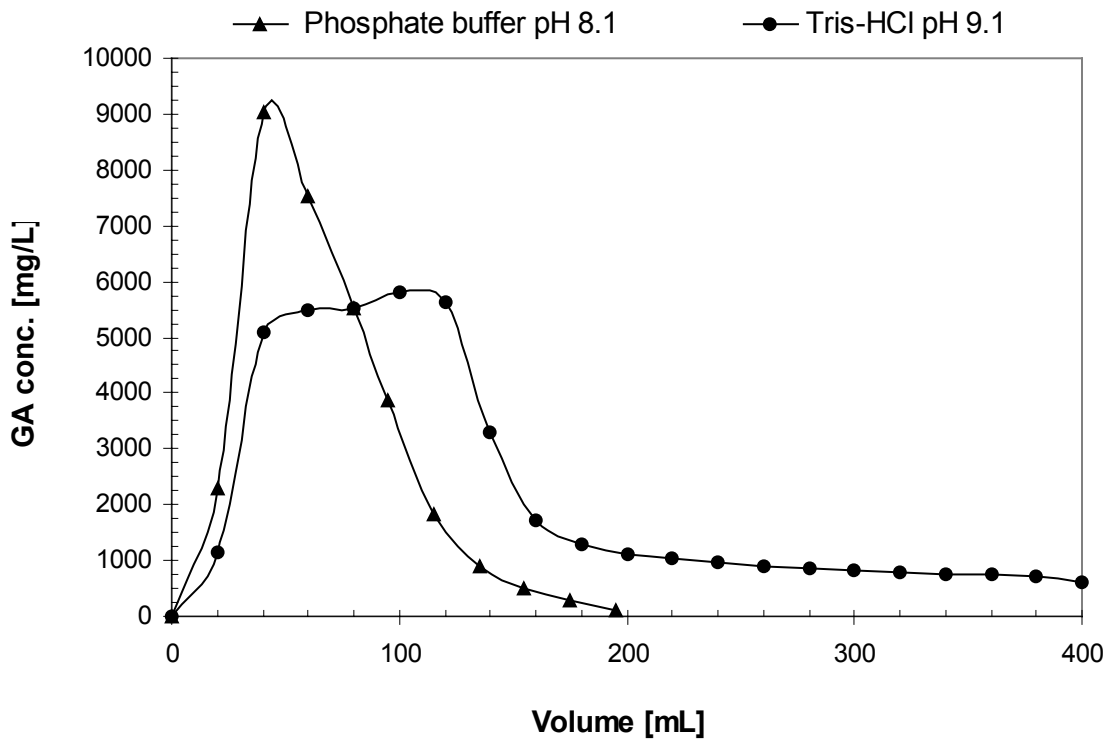


Fig. 23: Influence of pH on the galacturonic acid elution

3.1.7. Discussion

In this work, the research was focussed on the study of the low-priced renewable material extracted sugar-beet pulp as a source of the high-priced product galacturonic acid.

Commercial preparations, instead of purified enzymes, were selected with respect to a future industrial application. After a detailed enzyme screening, Pectinex®100L from Novo Nordisk, was selected for this purpose, due to the high release of galacturonic acid at short retention times. Pectinex®100L hydrolyses with high productivity pectin substrates to acid (galacturonic acid) and neutral (arabinose and galactose) monomers and oligomeric compounds in one unique set of experimental conditions. The pectin breakdown occurs with a low degradation of cellulose in accordance with the enzyme mixture low cellulolytic activity. With respect to the aim of this work only the aspect regarding the release of galacturonic acid was studied.

The enzymatic formation of galacturonic acid from sugar-beet pulp succeeds only when the cell-wall pectin of this substrate is extracted. For this purpose, the cell-wall has to be digested by heating for winning solubilised pectin, which can then be enzymatically treated [33]. This pre-treatment provides highest yields at elevated temperatures under pressure with the addition of aggressive reagents such as sulphuric acid. Based on economical considerations, the extraction of pectin was established in this work to succeed in water without addition of further chemicals at low energy input, that present equally commercial and environmental advantages.

In order to increase the production of galacturonic acid from pectin several parameters, such as pH and ionic activators, sodium and calcium cations, were investigated.

The influence of pH on the exo-polygalacturonase activity was examined in order to define the best conditions for the subsequent kinetic studies. Under the experimental conditions, the catalytic activity achieved the maximum at pH 3.8 and 50°C.

The effect of sodium and calcium cations on the enzymatic activity was investigated by supplementing the reaction medium with NaCl and $CaCl_2$. Neither Na^+, nor Ca^{2+} have enhanced or somehow influenced the exo-polygalacturonase activity.

Since sugar-beet represents a very complex substrate with a varying, non-standardisable composition, the kinetics of the enzyme mixture were studied at first with citrus- and sugar-beet pectin as model substrates. The degradation of the citrus- and sugar-beet pectin was carried out in acetate buffer (pH 4) and the decomposition of extracted sugar-beet pulp in water (pH 4.4).

On the basis of experimental results from the substrate kinetic series, the apparent kinetic parameters for both substrates, citrus- and sugar-beet pectin, were calculated with classical linearisation methods (Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee). From these studies, the mathematical description of the more complex reaction kinetics of the galacturonic acid release was developed (equation 4 is described at section 3.1.5.2).

The kinetic constants calculated with the three conventional methods were used in the proposed and developed mathematical model for computer simulation study. Based on these calculations it can be concluded, that the substrate inhibition is not relevant, but a strong product inhibition is given by galacturonic acid. This was found for all pectin substrates. The computed kinetic parameters are summarised in Tab. 9.

Tab. 9: Kinetic parameters for the conversion of the three pectin substrates to galacturonic acid

Parameter	PGA content [%]	K'_M [g/L]	r_{max} [g/(L×min)]	K'_{IP} [mmol/L]
CP	74.0	3.5	1.70×10^{-1}	5.4
SBP	41.0	3.0	1.67×10^{-1}	0.8
ESB	4.8	25.0	5.30×10^{-2}	2.6

For comparison of the enzyme mixture activity regarding the different substrates the different polygalacturonic acid content of each substrate should be considered. The degradation of citrus- and sugar-beet pectin takes place very fast, with the maximum yield achieved already after 2 h. The decomposition of the extracted sugar-beet pulp is slow, lower with a factor more than three compared to the other two reference substrates, achieving the maximum yield after 24 h. The yield of the enzymatic conversion depends mainly on the enzyme activity and the accessibility of the substrate. The complex matrix of the sugar-beet pulp fibres may hinder the degradation of pectin, as *Voragen et al.* previously described [54]. Therefore an efficient thermal pre-treatment, as described in section 3.1.4, is essential for a better enzymatic conversion.

The results of the kinetic series revealed that the parameters obtained on model substrates were not closely related to the degradation of the extracted sugar-beet pulp. As *Walter et al.* reported, the relative activities of the exo-polygalacturonase with various pectin sources as substrates can vary up to tenfold [8]. Nevertheless, the equation developed in this work, describes quite well the kinetics of the galacturonic acid formation from pectin. But due to the disregarding of the influence of several parameters on the kinetics, such as by-products, pH and viscosity, the experimental results may not be always in perfect agreement with the computed results.

Variations of the pH were notified both in buffered and non-buffered reaction medium. The decrease depends mainly on the substrate concentration, on the galacturonic acid concentration added in the product inhibition experiment series and on the product quantity released in the reaction. By the action of pectin acetylesterases, acetic acid is released in the medium consequently influencing the pH. The decrease of the pH during the reaction could be a favouring factor for the attack of the different enzymes present in the mixture. According to *Versari et al.*, the complete breakdown of pectin could be achieved only if the different types of enzymes are present in a correct proportion [12], but detailed information about the enzyme preparation composition were not available.

A good separation of the galacturonic acid from the other compounds of the reaction mixture was achieved by means of preparative cation exchange chromatography, whereas the concentrating of the product occured successfully by using preparative anion exchange chromatography.

Due to the potential for industrial application, a system for the production of galacturonic acid on technical scale was modelled as illustrated in Fig. 24. In this model-system the removal of galacturonic acid in a unit coupled to or integrated in the enzymatic reactor is possible. So, negative effects of product inhibition could be overcome.

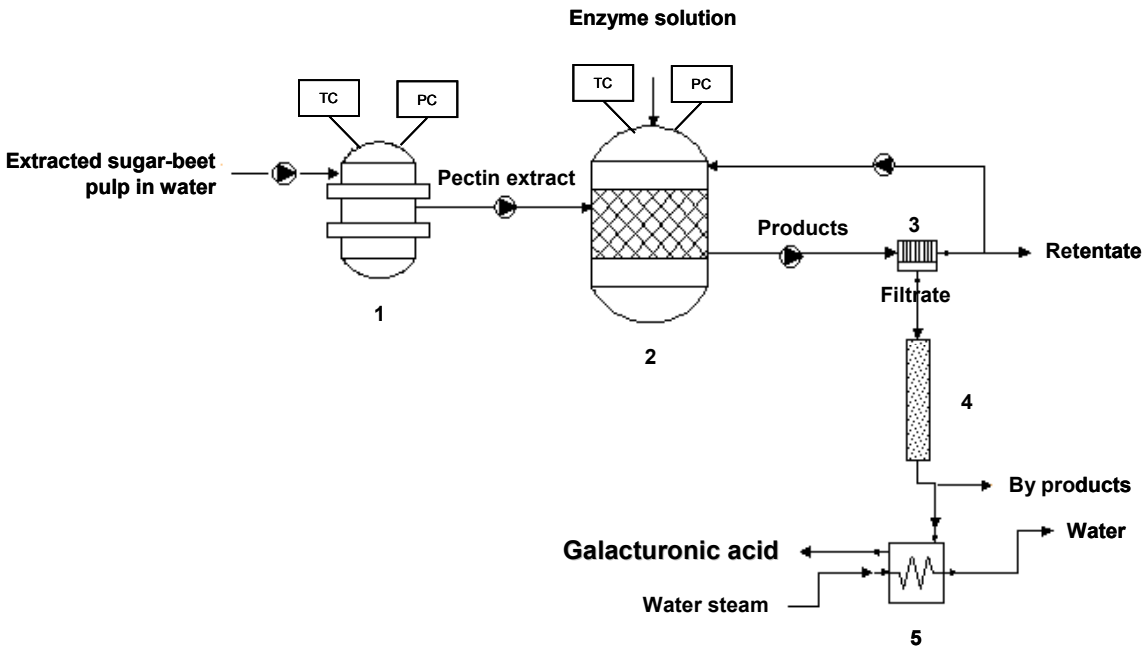


Fig. 24: Designed model-system for the production of galacturonic acid on technical scale (TC temperature control unit, PC pressure control unit)

The process steps of the designed system are as follows:

1. Thermal extraction of pectin from extracted sugar-beet pulp
2. Enzymatic galacturonic acid formation with the pectin extract as substrate
3. Separation of the pulp residues by filtration
4. Separation of galacturonic acid by ion exchange chromatography
5. Concentrating of the product (ion exchange chromatography, evaporation)

Concluding, this work pointed out an efficient pathway for the production of galacturonic acid (and even of other monomeric and oligomeric compounds) from extracted sugar-beet pulp. For this purpose, biotechnological (enzymatic catalysis) and chemical (extraction, separation and concentration) uncomplicated techniques were suitably integrated with regard to economical and environmental aspects.

3.2. Microbial synthesis of the sucrose analogue galactosyl-fructoside from sucrose

The chemical synthesis of sucrose analogues is extremely complex and the conventional enzymatic pathways with Leloir glycosyltransferases are disadvantageous due to the requirement of expensive nucleotide activated sugars as substrates. On the contrary, the sucrase type enzymes, developed and used in industry, surmount these difficulties by utilising relatively cheap substrates, as sucrose providing an economic feasible pathway for the sucrose analogues synthesis.

The basis for the synthesis of sucrose analogues from sucrose by means of sucrases and transferases, respectively fructosyltransferases is represented by the high energy of the glycosidic linkage of sucrose. The standard free-energy change accompanying the sucrose hydrolysis has been estimated to be –6600 cal/mol (-27.3 kJ/mol). This high value enables the sucrose analogues synthesis to occur by ensuring the energy supply demanded for the transfructosylation step [55].

3.2.1. Fructosyltransferase screening

Several commercial enzymes and enzyme preparations, summarised in Tab. 10, were initially screened for their ability to synthesise sucrose analogues by fructosyltransfer from sucrose in the presence of galactose, mannose and xylose as acceptors.

Tab. 10: Enzyme preparations for FTF screening

Enzyme (EC Number)	Source
Invertase (EC 3.2.1.26)	*Saccharomyces cerevisiae* [56]
Pectinex ULTRA SPL	*Aspergillis aculeatus* (Novo Nordisk) [40], [41]
Endofructosyltransferase	*Aureobasidium sp*. [42]
Levanfructotransferase	*Arthrobacter ureafaciens* K2032 [43]
Levansucrase (EC 2.4.1.10)	*Zymomonas mobilis* ZM1 (ATCC 10988) [57]
Fructosyltransferase (EC 2.4.1.162)	*B. subtillis* NCIMB 11871 [24], [44]

The first five enzymes showed excellent polymerising properties leading to FOSs with different polymerisation degrees (from trimer 1-kestose up to levan), detected with the HPAEC-PAD system. Furthermore, the chromatograms showed that no reaction with any of the three acceptors occured in the reactions catalysed by these enzymes.

The only FTF, which showed the transfructosylation property wanted was the enzyme produced by *B. subtilis* NCIMB 11871 [24]. The reaction with galactose as acceptor was successful leading to a single disaccharide product, galactosyl-fructofuranoside or galactosyl-fructoside (abbreviated Gal-Fru and found in literature also as galactosyl-fructose, galactosucrose and galsucrose). Oligo- or polymeric compounds were not detected. Therefore, all subsequent experiments were performed with this enzyme.

This FTF was isolated in 1989 by *Cheetam et al.*, from a food-approved microorganism, *B. subtilis* NCIMB 11871, was patented and has been recognised as being a novel type of FTF, which was given the number EC 2.4.1.162. The enzyme recommended and systematic names are as follows: aldose β-D-fructosyltransferase and α-D-aldosyl-β-D-fructoside:aldose 1-β-D fructosyltransferase respectively.

In disagreement with *Cheetam et al.*, which enumerated for this enzyme, in the list of acceptors (from methanol and glycerol to oligosaccharides such as kestose and trehalose) the aldoses mannose and xylose, no reaction with these two compounds was identified. The influence of the bacterial cultivation conditions may be considered to explain this divergence. Moreover, the enzymes may vary in the efficiency to perform this reaction with different acceptors. Consequently, the reaction may take place but with a very low yield for mannosyl-fructoside or xylosyl-fructoside, low yields that correspond to concentrations situated under the detection limit of the analytical tools utilised.

The FTF characterisation, separation and structural analysis of the Gal-Fru are presented subsequently.

3.2.2. *B. subtillis* NCIMB 11871 culture

The culture growth was studied with a liquid salt minimal mineral medium containing 1% sucrose as carbon and energy source and furthermore with the same medium enriched with 0.1% (w/v) yeast extract (see section 2.12) [45].

After a lag-phase of 15 h, a rapid exponential phase (5 h) followed, as illustrated in Fig. 25. The extracellular FTF was expressed in the supernatant already in the lag-phase of cell growth, reached the maximum activity in the stationary phase between 30 and 42 h cultivation time, followed by a rapid decrease.

The highest biomass growth rates were achieved with the medium supplemented with yeast extract (4.4 g/L after 50 h cultivation time), nearly twice as high as for the first medium (2.5 g/L). To the contrary, the FTF showed only half of the activity detected for the first medium in the supernatant: after 36 h fermentation, 108 U/L compared to 238 U/L

for the yeast extract free medium. According to these results, all subsequent fermentations were performed with the salts minimal mineral medium containing 1% sucrose without yeast extract addition.

It was noticed that often during the cultivation, the medium has modified its colour from milky transparent to lightly grey or even, in some cases, black. This colour change was attributed to a black pigment, that was also reported by *Cheetam et al*. [24]. In all fermentation series performed in this work, this "pigment" positively affected the activity of the FTF, even if in the literature was mentioned that it has no influence. The supernatants which contained the black pigment revealed a FTF activity up to 20% higher compared to those without pigment.

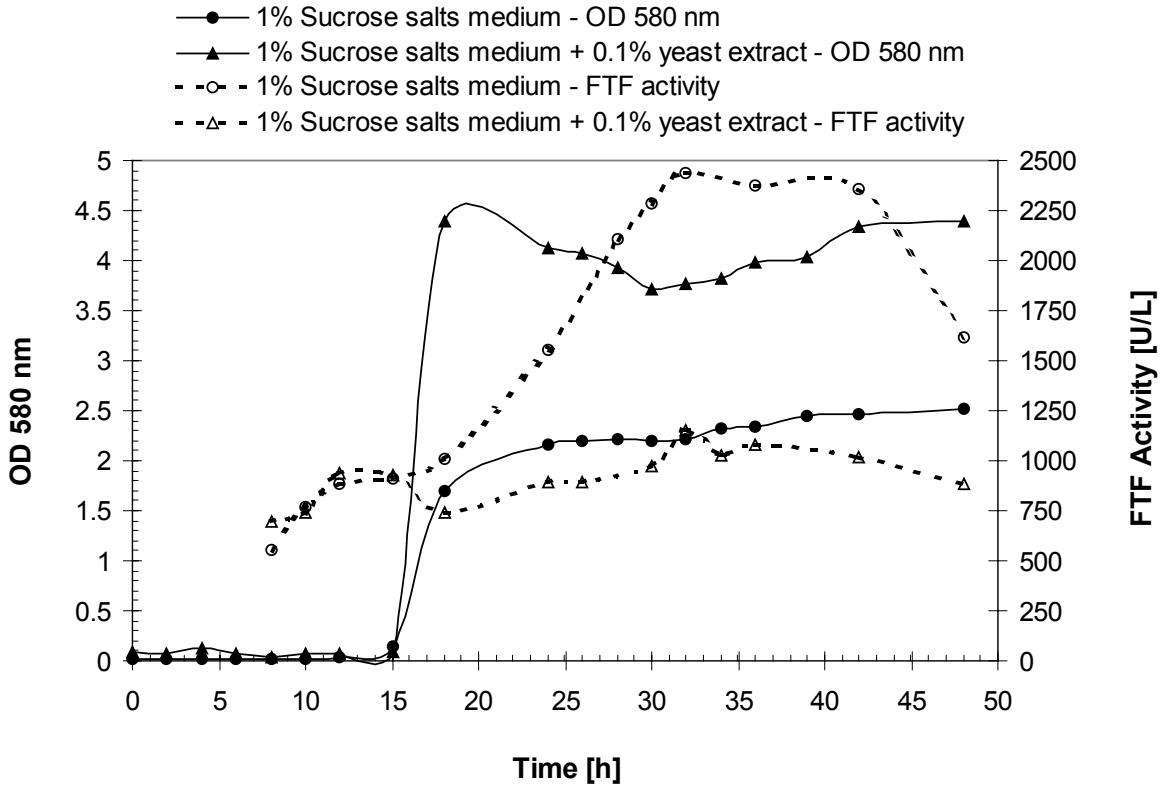


Fig. 25: *B. subtillis* NCIMB 11871 culture growth and FTF activity

The protein content of the supernatants was estimated to be on average of 0.1 mg per mL (Bradford method).

3.2.3. pH and temperature influence on fructosyltransferase activity

The influence of pH and temperature was examined in order to define the best conditions for the synthesis of Gal-Fru. According to the literature, the parameters for maximum activity of purified FTF from *B. subtilis* NCIMB 11871 are 30°C and pH values of 5 and 5.4 [24]. The optimum parameters of several bacterial and plant FTFs used for FOS large scale production, are reported to be in a range of pH of 5-6.5 and 50-60°C [18].

For the conditions studied (see experimental section 2.16), the catalytic activity achieved the maximum at pH 6 (Fig. 26) and at 50°C (Fig. 27), representing a maximal specific activity of 24 U/mg total protein, equivalent to an activity of 2400 U/L supernatant. After 6 h reaction, for a pH value of 8 (at 30°C) and for a temperature of 60°C (at pH 6), the relative activity loss was of about 40% and 50% respectively.

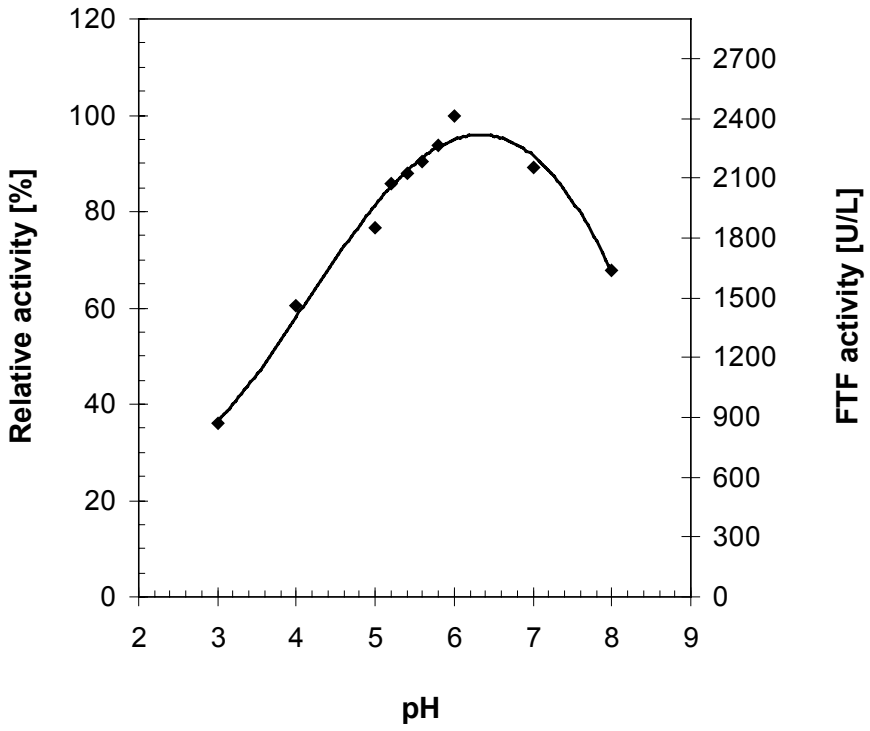


Fig. 26: Effect of pH on FTF activity

With regard to these results, all experimental series were performed at pH 6 and 50°C, to ensure the maximal FTF activity.

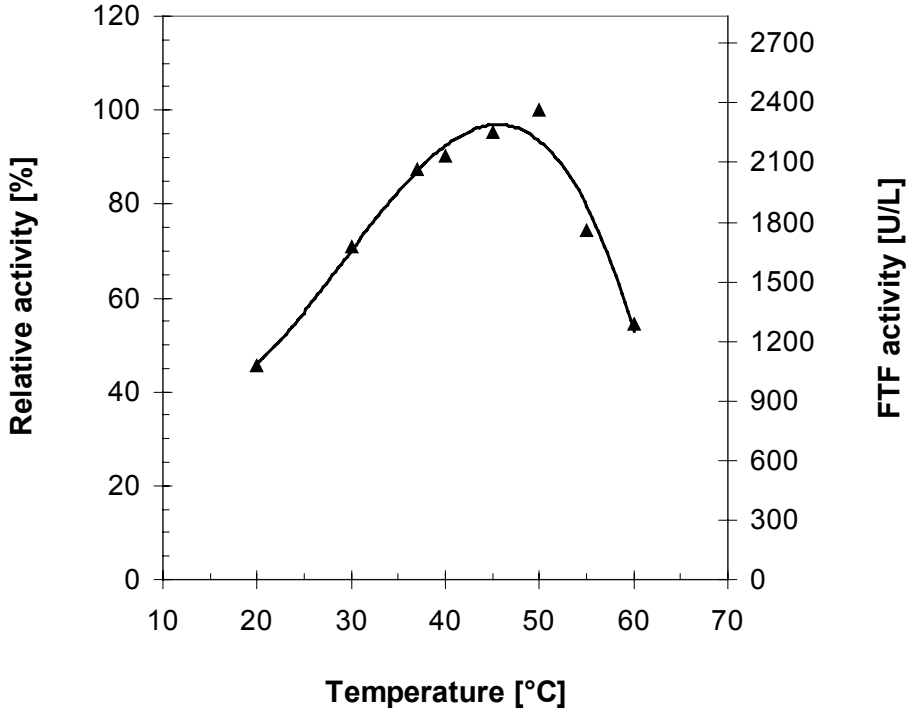


Fig. 27: Effect of temperature on FTF activity

It has to be mentioned that the enzyme, precisely the FTF supernatant is tolerant to physical stress. By storage at 4°C, no loss of activity could be detected within six month.

3.2.4. Activation energy

On the basis of the studies concerning the temperature dependency, the initial reaction rate (*r*) was calculated for each temperature. The data resumed in Tab. 11 were plotted as ln *r* versus 1/K giving as linear regression the slope of $-E_a/R$ as depicted in Fig. 28.

According to the Arrhenius equation the activation energy for the reaction catalysed by FTF was calculated to be 21.4 kJ/mol, with a correlation coefficient of $R^2 = 0.97$.

This is the first time when the activation energy for this reaction was calculated, thus no comparison with other FTF was possible. The values of the E_a for many enzymatic catalysed reactions are in the range of 40-50 kJ/mol [38]. It must be underlined that the value of E_a for FTF reaction was calculated for the culture supernatant and not for pure FTF. Therefore a reason to be taken into account for the low activation energy could be the influence affected by the action of other enzymes or proteins present in the supernatant. These proteins may show side-activities influencing the product

concentrations, leading to an apparent value of the E_a, resulted as a sum of Gal-Fru formation by FTF and destruction by other enzymes.

Tab. 11: FTF reaction rates at different temperatures (after 1 h reaction time)

T [°C]	Initial reaction rate *r* [mmol Glu/(L×min)]	Relative activity [%]
20	1.08 ± 0.1	50.2
30	1.21 ± 0.1	55.9
37	1.71 ± 0.2	79.2
40	1.88 ± 0.3	87.4
45	1.93 ± 0.1	87.7
50	2.16 ± 0.2	100.0
55	1.92 ± 0.4	89.0
60	1.63 ± 0.2	75.5

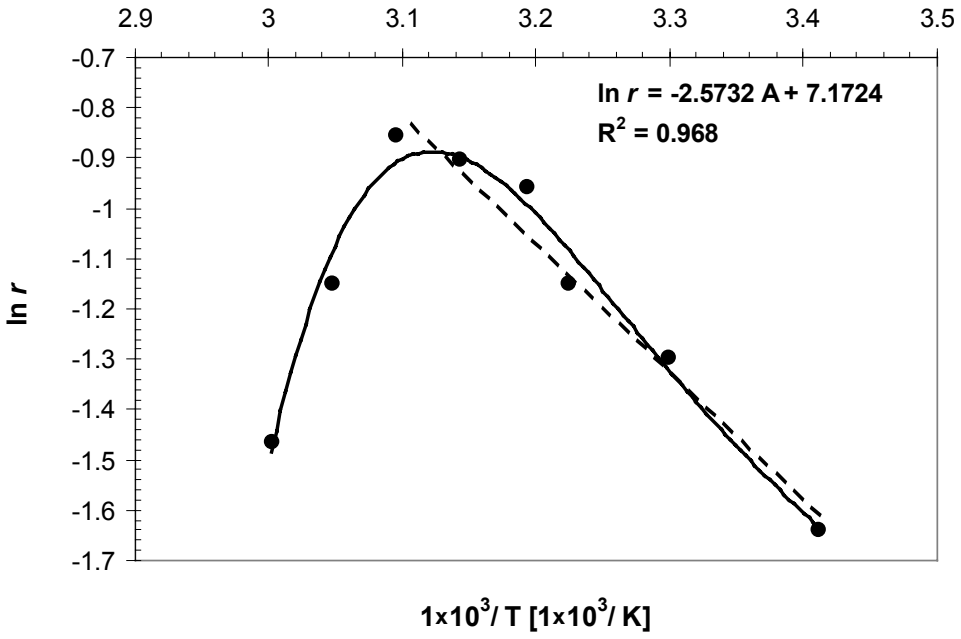


Fig. 28: Arrhenius plot for FTF

As shown in Fig. 28, for higher temperatures after one hour reaction time, the inactivation by denaturation superimposes the increasing rate of the reaction. Therefore, the inactivation of the FTF rapidly occurs at temperatures above 50°C.

3.2.5. TLC of the transfructosylation reaction

For qualitative and quantitative determination of sucrose consumption and product formation, three analytical methods were used for monitoring the FTF reaction: HPAEC-PAD, IC and TLC.

As detected on the TLC plate, the spots with the lowest response factors (R_f) are for the polymers synthesised in the dextransucrase (DS) reaction, followed by the tetramer stachyose, trimer raffinose, the dimers melibiose, new sucrose analogue Gal-Fru and sucrose and the monosaccharides, galactose, glucose and fructose. With the chosen solvent system all mono- and disaccharides, as well as polymers were very well separated.

The specific colour, dependent on the saccharide structure and the R_f value of each sugar are summarised Tab. 12.

Tab. 12: R_f values and colour of the carbohydrates implied in the FTF and DS reaction

Carbohydrate	Colour	R_f
Dextran	Black	0 (on the start line)
Oligosaccharides	Grey-black	(0.06 – 0.3)
Stachyose	Black	0.12 ± 0.02
Raffinose	Black	0.20 ± 0.02
Melibiose	Black	0.23 ± 0.03
Galactosyl-fructoside (Gal-Fru)	Violet-red	0.42 ± 0.03
Sucrose	Violet-red	0.50 ± 0.05
Galactose	Grey-black	0.55 ± 0.02
Glucose	Grey-black	0.65 ± 0.03
Fructose	Red-violet	0.72 ± 0.03

3.2.6. Substrate specificity

Apart from sucrose, two higher homologues of sucrose, the trimer raffinose and the tetramer stachyose (for structure see Fig. 6 from section 1.2), were tested as fructosyl donor, in the reaction catalysed by FTF.

With all the three substrates tested, the synthesis of Gal-Fru occured successfully as illustrated in Fig. 29, Fig. 30 and Fig. 31. Fig. 51 from the Appendix section stands as example for the chromatogram related to the TLC plate illustrated in Fig. 29.

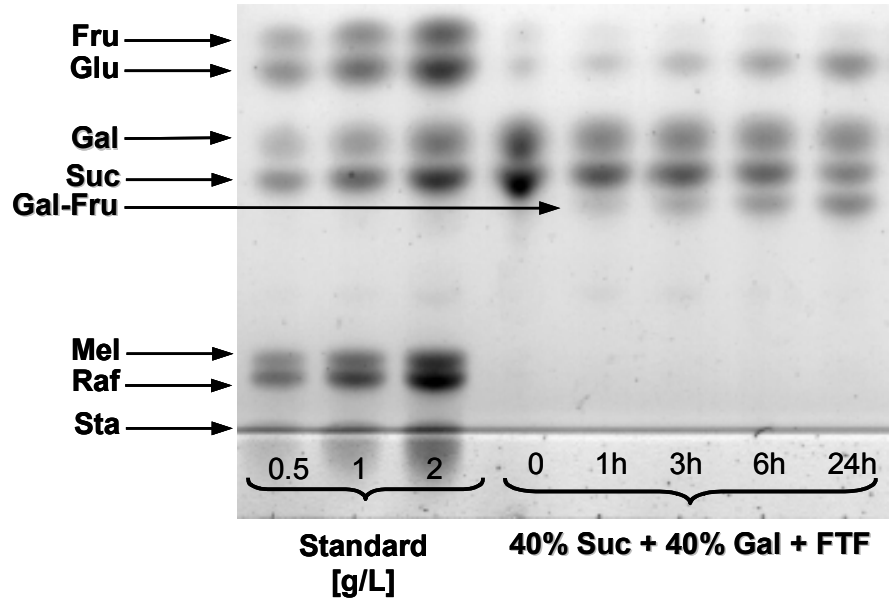


Fig. 29: TLC analysis of the FTF reaction with sucrose as substrate

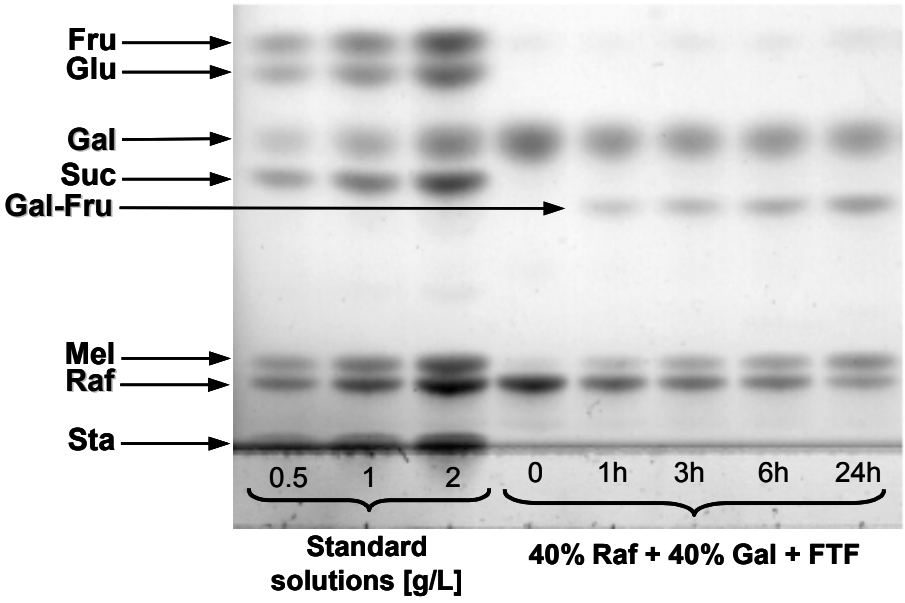


Fig. 30: TLC analysis of the FTF reaction with raffinose as substrate

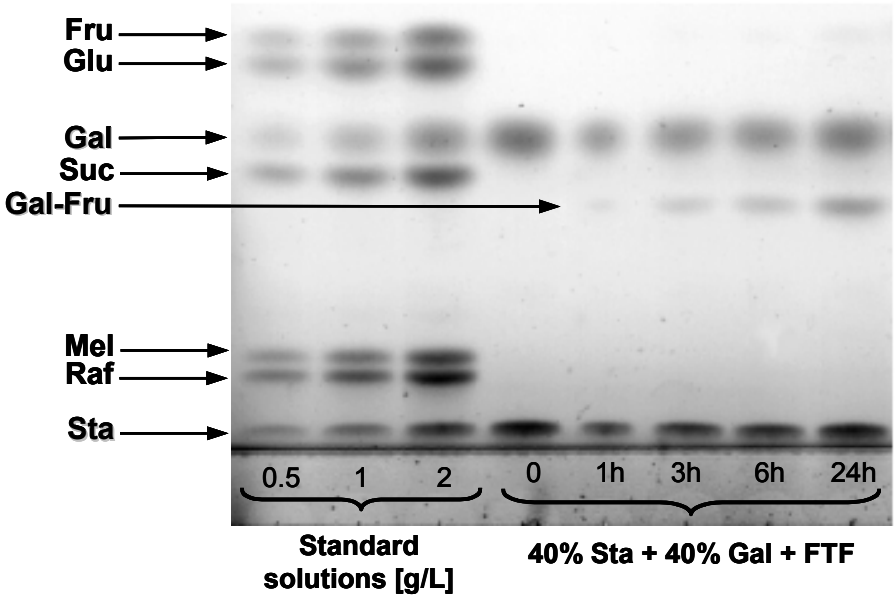


Fig. 31: TLC analysis of the FTF reaction with stachyose as substrate

It was observed by *Cheetam et al.* [24], that carbohydrates based on sucrose with an 1-2 linkage, are an absolute requirement for the FTF transfructosylation reaction. The use of raffinose as fructosyl donor in a levansucrase (from *Aerobacter levanicum*) catalysed transfer reaction, was previously reported by *Feingold et al.* for synthesis leading to sucrose analogue β-D-fructofuranosyl-α-D-xyloside (Xyl-Fru) and to galsucrose [28].

In the case of the trimer and tetramer as substrates the transfructosylation reaction can be described by the equation presented below:

$$\text{Glu-(Gal)}_n\text{-Fru} + \text{Gal} \xrightarrow{\text{FTF}} \text{Glu-(Gal)}_n + \text{Gal-Fru}$$

Accordingly, when raffinose (n=1) and stachyose act as substrates (n=2), the two end products (apart from Gal-Fru) are the disaccharide melibiose O-α-D-galactopyranosyl-(1-6)-α-D-glucopyranoside and the trisaccharide O-α-D-galactopyranosyl-(1-6)-O-α-D-galactopyranosyl-(1-6)-α-D-glucopyranoside (structure not analysed).

Based on economic considerations, the Gal-Fru synthesis was consequently performed with the low-cost substrate sucrose, despite the fact that on this pathway an additional step is required (represented by the reaction with dextransucrase for removing the unreacted sucrose).

3.2.7. Synthesis of Gal-Fru

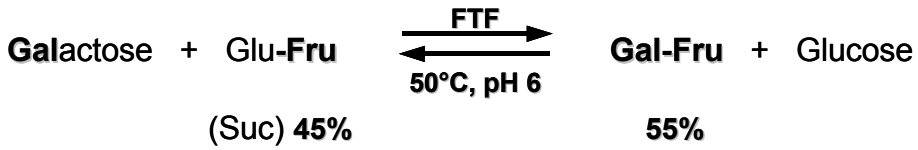
The transfructosylation reaction from sucrose as fructosyl donor was studied with regard to Gal-Fru synthesis as described in section 2.17. The data obtained in all three experimental series summarised in Tab. 13, indicated that the transfer reaction occured with a maximal 55% Gal-Fru average yield and correspondingly 55% sucrose conversion after 24 h.

Tab. 13: Kinetic series results with FTF

Kinetic series	*r* [mmol Glu/(L×min)]	Sucrose conversion	
		after 6 h [%]	after 24 h [%]
40% Suc + 15% Gal	1.24	12	48
40% Suc + 30% Gal	0.95	15	58
40% Suc + 60% Gal	0.47	31	61
20% Suc + 5% Gal	0.77	25	54
20% Suc + 10% Gal	0.53	27	58
20% Suc + 20% Gal	0.59	32	67
20% Suc + 40% Gal	0.53	24	29
5% Suc + 40% Gal	0.48	44	64
10% Suc + 40% Gal	0.72	47	53
20% Suc + 40% Gal	1.07	20	47
40% Suc + 40% Gal	1.27	24	49
60% Suc + 40% Gal	1.14	30	46

It must be mentioned that all reactions were performed at the optimal temperature of 50°C, that could lead to thermo inactivation of the enzyme. But when the same reactions series was carried out at 30°C equivalent results were achieved. For this reason, the aspect of the thermo inactivation of FTF can be excluded and the hypothesis of the equilibrium reaction can be confirmed. The equilibrium of the transfructosylation reaction catalysed by this FTF was not reported in the literature before.

It can be assumed, that the equilibrium establishes between the forward and reverse reaction: in the forward reaction the fructosyl residue is transferred to the acceptor galactose, subsequent to Gal-Fru synthesis and in the reverse reaction the fructosyl residue is transferred to the glucosyl group resulting in sucrose formation. A schematically representation of the transfructosylation equilibrium reaction is presented below:



where 45% refers to the percentage of the unreacted sucrose.

A comparable equilibrium reaction, was mentioned for the substrate-acceptor system sucrose-melibiose in the reaction catalysed by levansucrase from *Aerobacter levanicum* [28].

In addition, it may be supposed that the by-product of the fructose transfer reaction, the glucose that represents also a potential acceptor might compete with galactose leading to reformation of the starting substrate. In this case, a solution to shift the reaction equilibrium to the Gal-Fru formation may be the “on-line” removal of glucose by reaction engineering or enzymatic pathways (for instance by conversion of glucose into fructose by addition of glucose isomerase) [44].

In the performed kinetic series, the highest initial reaction rate (1.27 mmol glucose/L) was calculated in the reaction with 40% sucrose and 40% galactose that represents a molar ratio of 1/1.9 for substrate/acceptor. At a constant sucrose concentration of 40% and increasing acceptor concentrations, for 60% galactose the initial reaction rate (*v*) decreases significantly with more than 60% (calculated taking the highest initial rate of 1.27 mmol/L as 100%).

In the series with 20% sucrose, with increasing galactose concentrations low initial reaction rates, sucrose conversion and Gal-Fru yields (after six hours) values were calculated. That confirms the recognised fact that the acceptor reaction occurs at both high substrate and acceptor concentrations, in order to achieve substantial yields at short retention times.

As shown in Tab. 13, for 40% acceptor concentration and increasing sucrose concentrations, the initial reaction rate rises up to 40% sucrose concentration, after that decreases slightly. In the reaction with 60% sucrose, a 10% decrease of the initial reaction rate can be calculated. This is rather insignificant compared to the drastic decrease of more than 60% determined for 40% sucrose and 60% galactose.

According to these results higher substrate concentrations than those for the acceptor have no considerable influence on the initial reaction rate and yield of Gal-Fru. On the contrary, at increased acceptor concentrations the transfructosylation reaction occurs with low reaction rate that may indicate an inhibiting effect of galactose.

Tests with higher sucrose-galactose concentrations were not performed, since experimental handling problems appeared already in the experiments with 60% galactose and 60% sucrose. These were determined mainly by the limit of solubility of the reactants and viscosity of the reaction mixture.

On the basis of these results it can be concluded that the ratio of fructose donor to fructose acceptor is essential, as previously reported by *Cheetam et al.*. In disagreement to their studies that established a molar ratio donor to acceptor of 1 to 5 [24], in this work a ratio of 1 to 1.9 was found to be the most suitable for the Gal-Fru synthesis.

Except for the levansucrases from *B. subtilis* and *A. diazotrophicus*, no systematic reported kinetic studies of this type of enzymes were reported. Several earlier kinetic analysis of the transfructosylation reactions led to the conclusion that catalysis proceeds via a ping-pong mechanism. This involves the formation of a transient covalent fructosyl-enzyme intermediate which preserves the high energy of the glycosidic linkage of sucrose [55].

3.2.8. Fructosyltransferase concentrating by ultrafiltration

The *B. subtilis* NCIMB 11871 FTF, implied in this study, is until now poorly characterised. For this reason, three similar enzymes which are well described in literature were selected as model:

a. Levansucrase from *B. subtilis* (EC 2.4.1.10)

b. 1-SST: sucrose 1F-fructosyltransferase (EC 2.4.1.99)

c. 1-FFT: 1,2-β-Fructan 1F-fructosyltransferase (EC 2.4.1.100).

The molecular weight of the bacterial levansucrase and of both plant-tissue FTFs are reported to be 49.9 kDa [58], 65 kDa and respectively 72.8 kDa [26]. Therefore, the molecular weight cutt-offs (MWCOs) of the ultrafiltration membranes were chosen with consideration to the molecular weight of these three model enzymes. The tangential (cross-over) flow rate ultrafiltration was performed with a 30 kDa MWCO polyethersulfone membrane, whereas the frontal flow rate ultrafiltration was carried out both with 30 kDa and 50 kDa MWCO membranes.

The results of the two concentrating methods tested are summarised in Tab. 14. With no exception, whatever MWCO membrane was used (30 and 50 kDa) the FTF remained mainly in the retentate, the filtrates showing negligible FTF activity values (FTF activity ≤

0.1 U/mL).

Except for the 50 kDa RFF*, the calculated initial reaction rates and activities of the two 30 kDa retentates (RFF and RTaF*) are comparable to the activity of the FTF supernatant. In the case of the 50 kDa RFF, an increase of both initial reaction rate and activity values corresponding to more than 300% and 140% respectively, were determined.

Tab. 14: Results of the two types of flow ultrafiltration

Enzyme solution	Activity [U/mL] after 6 h	Protein [mg/mL]	Specific act. [U/mg]	Specific act. loss [%]
FTF supernatant	2.5	0.05	50.0	-
30 kDa RTaF*	2.7	0.19	14.2	71.6
30 kDa RFF*	2.6	0.06	43.3	13.4
50 kDa RFF*	3.5	0.19	18.4	63.2

Fig. 32 illustrates, for all the resultant retentates and filtrates, the corresponding glucose formation curves from which the values summarised in Tab. 14 are calculated. For an overall view, several IC overlaid chromatograms are exemplified in Fig. 52 and Fig. 53 at the Appendix section.

The concentrating by tangential flow ultrafiltration with the 30 kDa MWCO membrane and by frontal flow ultrafiltration with 50 kDa MWCO membrane of the FTF supernatant led to substantial increase of the protein content of about four-fold. However, apart from the 50 kDa RFF, the concentrating/purification step by ultrafiltration directed to no enhancement of the FTF specific activity. Therefore, it may be concluded that the presence of other proteins, enzymes or metabolic compounds contained in the FTF supernatant, which are separated by performing this procedure, exhibit no effect on the FTF activity.

* **RFF**: **R**etentate concentrated by **F**rontal **F**low rate ultrafiltration; **RTaF**: **R**etentate concentrated by **Ta**ngential **F**low rate

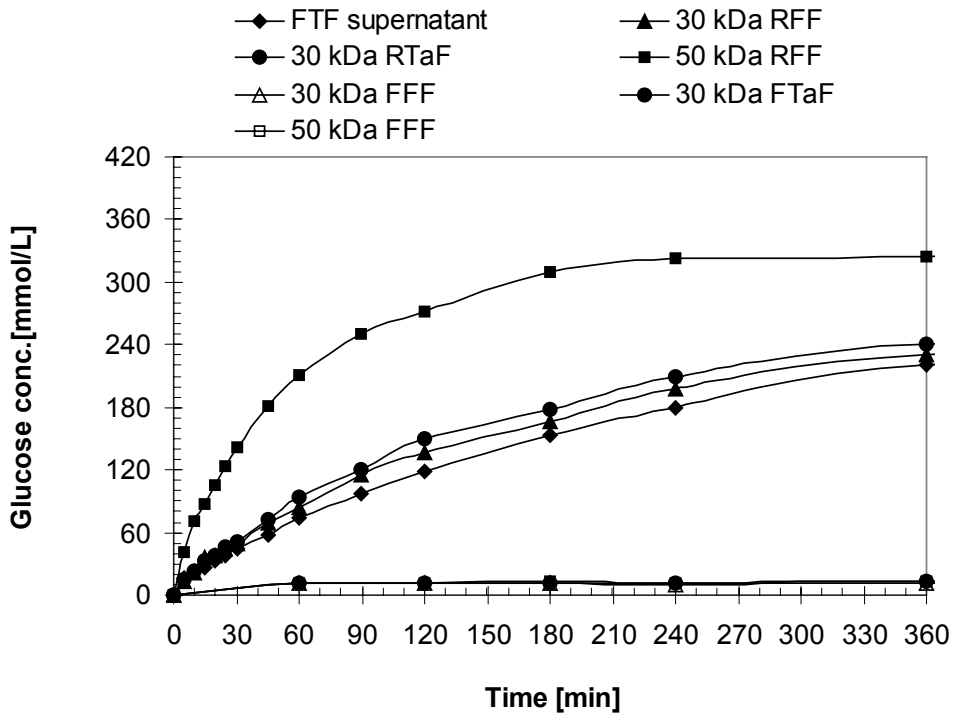


Fig. 32: Glucose release in the reactions with the FTF supernatant, the retentates and filtrates resulted by concentrating with the two ultrafiltration methods (FFF: Filtrate concentrated by **F**rontal **F**low rate ultrafiltration**; FTaF: F**iltrate concentrated by **Ta**ngential **F**low rate**)**

The specific activities of all retentates obtained by the two ultrafiltration methods decreased. The biggest loss, corresponding to almost 72% was calculated for the 30 kDa RTaF. An explanation of this important specific activity loss may be a high membrane adsorbance, in contradiction with the theoretical low binding properties of the membrane (as described by the provider) or a membrane fouling phenomenon. Similar results regarding the activity loss were reported by *Le Gorrec et al*., also for ulltrafiltration by tangential flow with various MWCO membranes (10, 50 and 100 kDa) of the FTF supernatants from *B. subtilis* NCIMB 11871 [59]. Their results indicated moreover that the FTF activity was found in the retentate after frontal and tangential filtration with 100 kDa MWCO membrane, thus the FTF should have a molecular weight higher than 100 kDa. This aspect is not surprisingly since the FTFs and levansucrases described so far in literature differ widely in characteristic properties. The main differences between these enzymes are related primarily to the molecular weight and secondly to the donor, acceptor and inhibitor specificity.

In agreement with *Le Gorrec et al*., *Song et al.* described a recombinant FTF from *Streptococcus salivarius* ATCC 25975 with a molecular weight of 102 kDa [60]. By the

characterisation of recombinant inulosucrase from *Leuconostoc citreum*, a cell-associated hybrid enzyme labelled by the authors as a "fructosyltransferase within a glycosyltransferase", the highest molecular mass for FTFs equivalent to 165 kDa was reported [61].

In all test performed with the FTF supernatant and the retentates resulted by ultrafiltration, not only the glucose release but the formation of Gal-Fru was also analysed. As shown in Fig. 33, the maximum area and consequently, also the highest concentration of Gal-Fru is achieved for the 50 kDa RFF, up to 180 minutes reaction time.

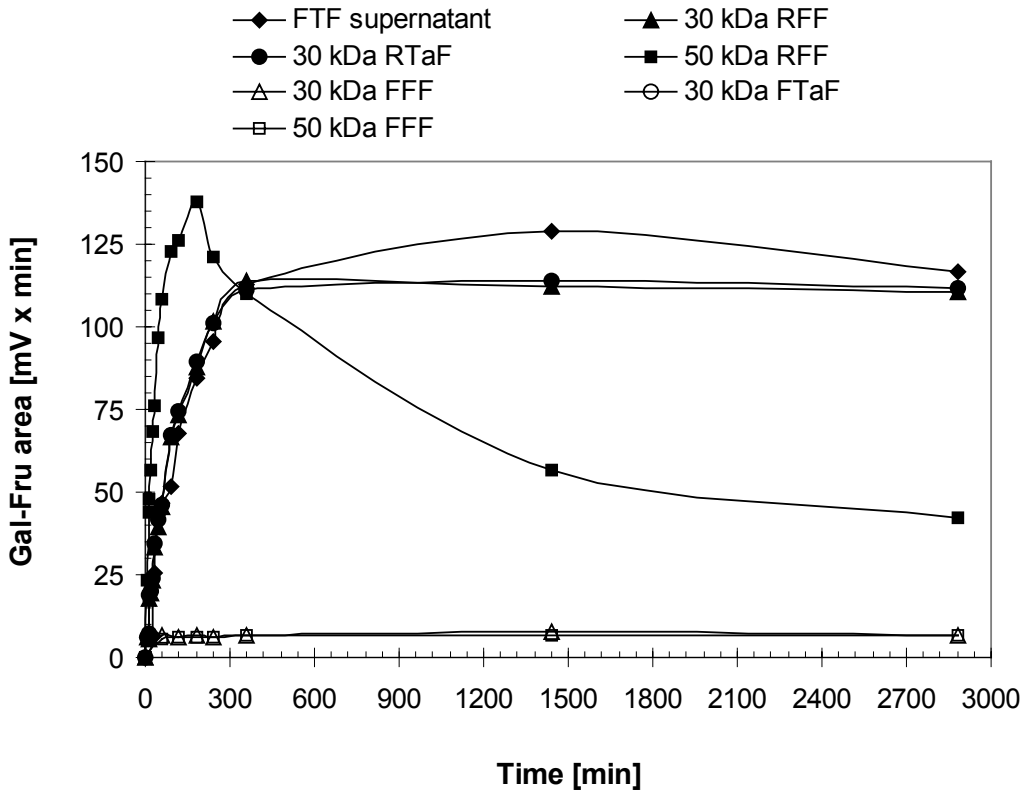


Fig. 33: Gal-Fru formation with the FTF supernatant, the retentates and the filtrates resulted by concentrating with the two ultrafiltration methods

After this time interval, the area of this sucrose analogue decreases drastically, resulting after 24 h in a concentration loss of about 70% (no more than 30% residual Gal-Fru concentration).

Simultaneously with the decrease of the Gal-Fru area, two peaks corresponding to oligomeric compounds are detected by IC analysis as depicted in Fig. 34.

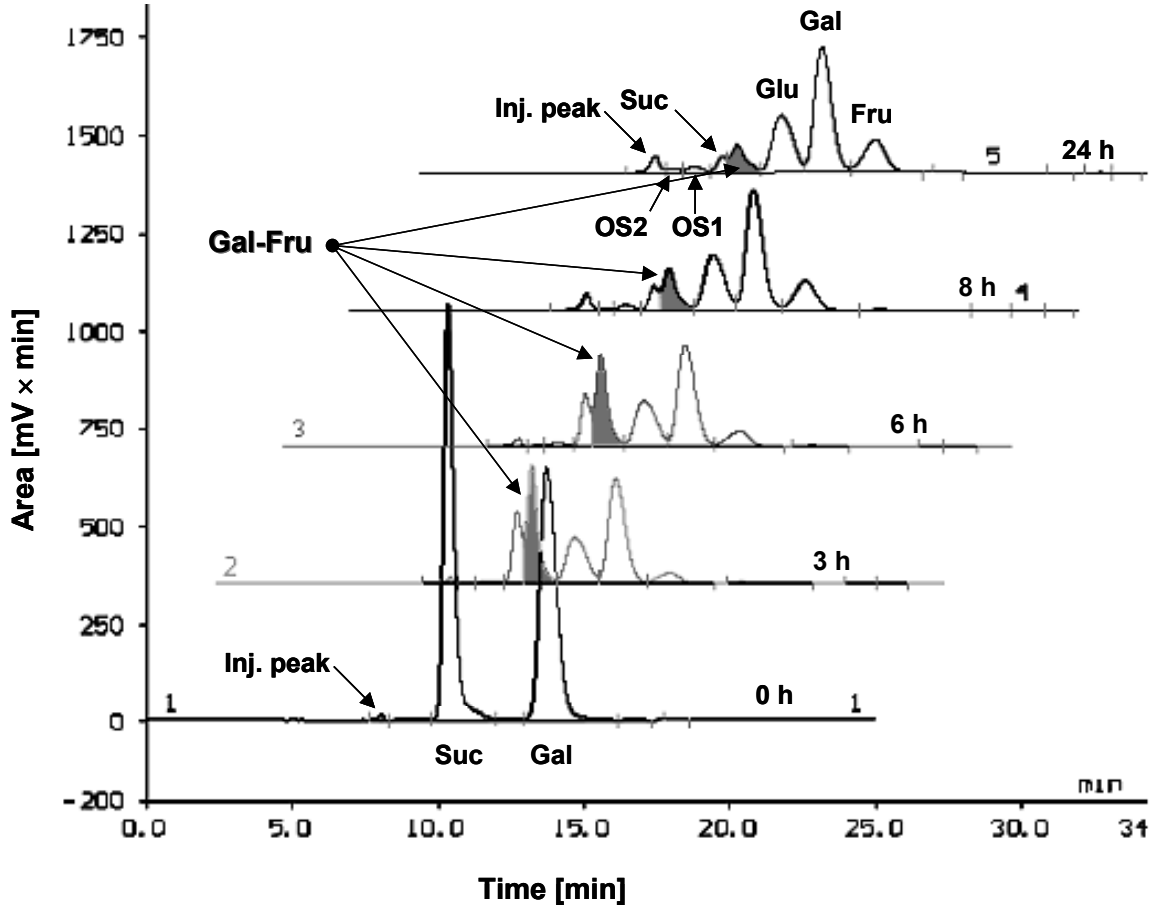


Fig. 34: IC chromatogram of the transfructosylation reaction with 50 kDa RFF

Based on the fact that the IC column is designed particularly for the separation of the mono- and disaccharides and not for oligomeric carbohydrates, it can be assumed that the number of oligomers may be higher than two. For the reason that from the polymerisation degree of five the column capacity is overcome, higher oligomers are eluted in a single peak corresponding to the injection peak. Therefore, a qualitative and quantitative determination of the oligomeric carbohydrates, having a polymerisation degree higher than five, is not achievable with the IC.

In the transfructosylation reaction catalysed by the 50 kDa RFF since the area of the injection peak increases, it can be concluded that the oligomers synthesised are more than two. Moreover, for the oligomeric compounds that are eluted in the injection peak it can be supposed to have a polymerisation degree higher than five.

The degradation of the sucrose analogue Gal-Fru in the reaction with the 50 kDa RFF is an unexpected behaviour that is not reported in the literature. Several hypothesises may

be formulated. By ultrafiltration with 50 kDa MWCO, it may be assumed that a factor having a molecular weight higher than 30 kDa but lower than 50 kDa, which influences the FTF activity, might pass through the membrane.

If the FTF possesses only one catalytic site both for transfructosylation and polymerisation two explanations might be considered. Firstly, the above mentioned factor could be an activator of the transfructosylation reaction, in whom absence the fructosyl transfer to the acceptor galactose may occur very slow and concurrently, the polymerising activity may be favoured. Secondly, this factor could be an inhibitor of the polymerising reaction. As result of its removal, the polymerisation activity may be enhanced leading to high polymerising reaction rates that may superimpose the transfructosylation reaction rates.

In the supernatant, the FTF may possibly be aggregated to other extracellular compounds, such as proteins, enzymes or carbohydrates, as usually for culture and protein solutions. For instance dextransucrase has a dextran chain attached to it, otherwise this enzyme is neither stable or active (the dextransucrase from *L. mesenteroides* presents 95% dextran attached) [29]. The reason for the high stability of the levansucrase from *B. subtilis* was reported to be caused by the aggregation of levansucrase molecules [59]. Furthermore, for levansucrases from *Pantoea agglomerans* and *Pseudomonas syringae pv. phaseolicola* the existence of similarly large aggregates bound together by sugar polymers, such as levan was also described [62]. These aggregated compounds may “camouflage” the polymerisation active catalytic site, when supposed that for transfructosylation and polymerisation the FTF exhibits two different sites. It may be assumed that consequent to the removal of the aggregated compounds, the steric hindrance could be overcome and the polymerisation site may remain exposed for the substrate.

The unusual behaviour of the 50 kDa RFF described above, can represent a challenging topic for future study. The determination of the oligomer types synthesised, if fructose homopolymers or heteropolymers (having the monosaccharides Fru, Gal and Glu as building blocks) and of the nature of their glycosidic bond, whether inulin, fructan or levan type is very motivating since it can lead to the discovery of novel products.

3.2.9. Sucrose hydrolysis by fructosyltransferase

The most important characteristic of the FTF from *B. subtilis* NCIMB 11871, that represented the mile-stone of this work for the synthesis of Gal-Fru, is that in the substrate acceptor reaction this enzyme lead to little, if any fructan or levan polymer formation. For several FTFs it was previously reported that sucrose is primarily consumed to produce

polymer, the acceptor reaction being secondary [24], whereas with the FTF studied in this thesis, substantial amounts of sucrose remain unreacted (45%) once the maximum yield of Gal-Fru has been achieved (equilibrium reaction as described in section 3.2.7).

When the FTF supernatant, the 30 kDa RFF and the 50 kDa RFF were incubated only with the substrate sucrose, water playing the acceptor role, the main compounds formed were fructose and glucose. However, several spots matching to oligomeric compounds could be detected both by TLC and IC analysis (IC chromatogram shown at Appendix section in Fig. 54). On the TLC plate illustrated in Fig. 35, near the three well-defined spots of sucrose and of the two major products, fructose and glucose, six new spots under sucrose can be identified.

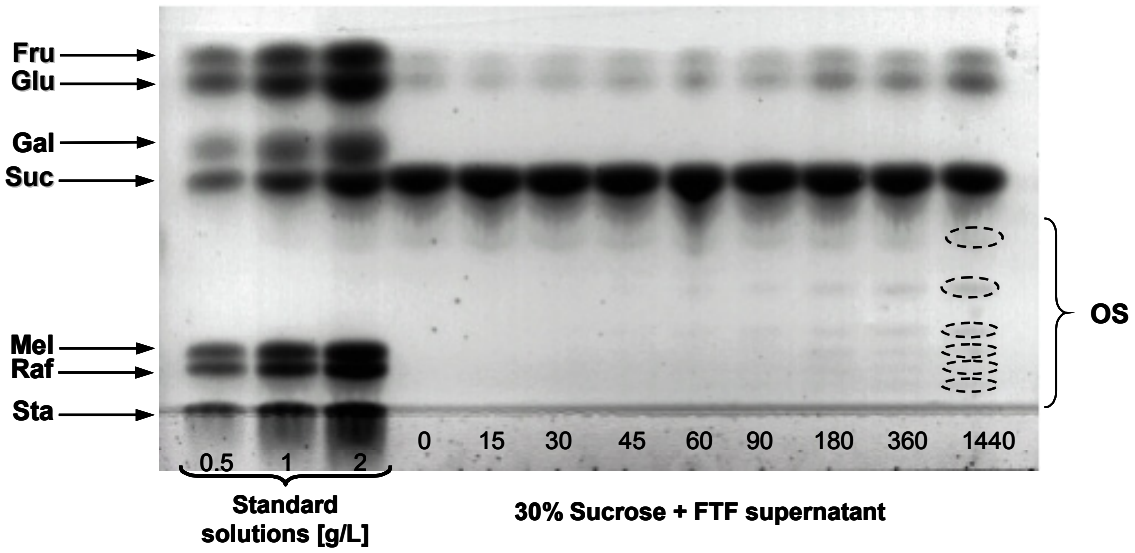


Fig. 35: Sucrose hydrolysis by FTF (time intervals in minutes)

The results summarised in Tab. 15 point out that the highest initial reaction rate is attained for the hydrolysis with the 50 kDa RFF and it can be evaluated that this value is almost four-fold lower compared to the one calculated for the fructosyl transfer reaction (for evaluation see Tab. 14). The hydrolysis initial reaction rate values for the FTF supernatant and 30 kDa RFF represent less than 50% of those for the transfructosylation reaction, with 40% sucrose-40% galactose.

Tab. 15: Sucrose hydrolysis results (FTF activity calculated after 6 h enzymatic reaction)

Enzyme solution	*r* [mmol Glu/(L x min)]		Activity [U/mL]	Specific activity [U/mg]
	30% Suc	40% Suc-40% Gal		
FTF supernatant	0.7	1.6	0.7	14
30 kDa RFF	0.7	1.8	0.8	13
50 kDa RFF	1.4	5.1	4.6	25

The hydrolytic activity of the 50 kDa RFF is with approximately 25% higher as the transfructosylation activity, while for the FTF supernatant and 30 kDa RFF the values are almost 70% lower. According to these results it can be concluded that in the absence of an acceptor, the sucrose hydrolysis catalysed by this FTF occurs relatively slowly.

The concentration of the educts and products of the hydrolysis reaction series are summarised in Tab. 16. The start concentration of sucrose was 30% equivalent to 877 mmol/L.

Tab. 16: Educts and products concentration by sucrose hydrolysis with FTF after 24 h

Enzyme solution	Concentration [mmol/L]					
	Sucrose consumption	Formation				
		Glu	Fru	OS 1	OS 2	Injection peak area [mV x min]
FTF supernatant	301	300	275	21	25	23
30 kDa RFF	333	355	325	30	28	43
50 kDa RFF	891	782	321	48	-	180

The results show that sucrose is “almost completely” hydrolysed at the end of the reaction with the 50 kDa RFF in agreement with its high activity. The balance for the sucrose consumed in the hydrolysis reaction catalysed by the 50 kDa RFF indicates a total hydrolysis. However, on the related chromatogram illustrated in Fig. 36 the peak for sucrose is still present. Because of this discrepancy, that could be caused by measurement experimental errors, the term “almost completely” related to the sucrose hydrolysis with the 50 kDa RFF was used in the context mentioned above.

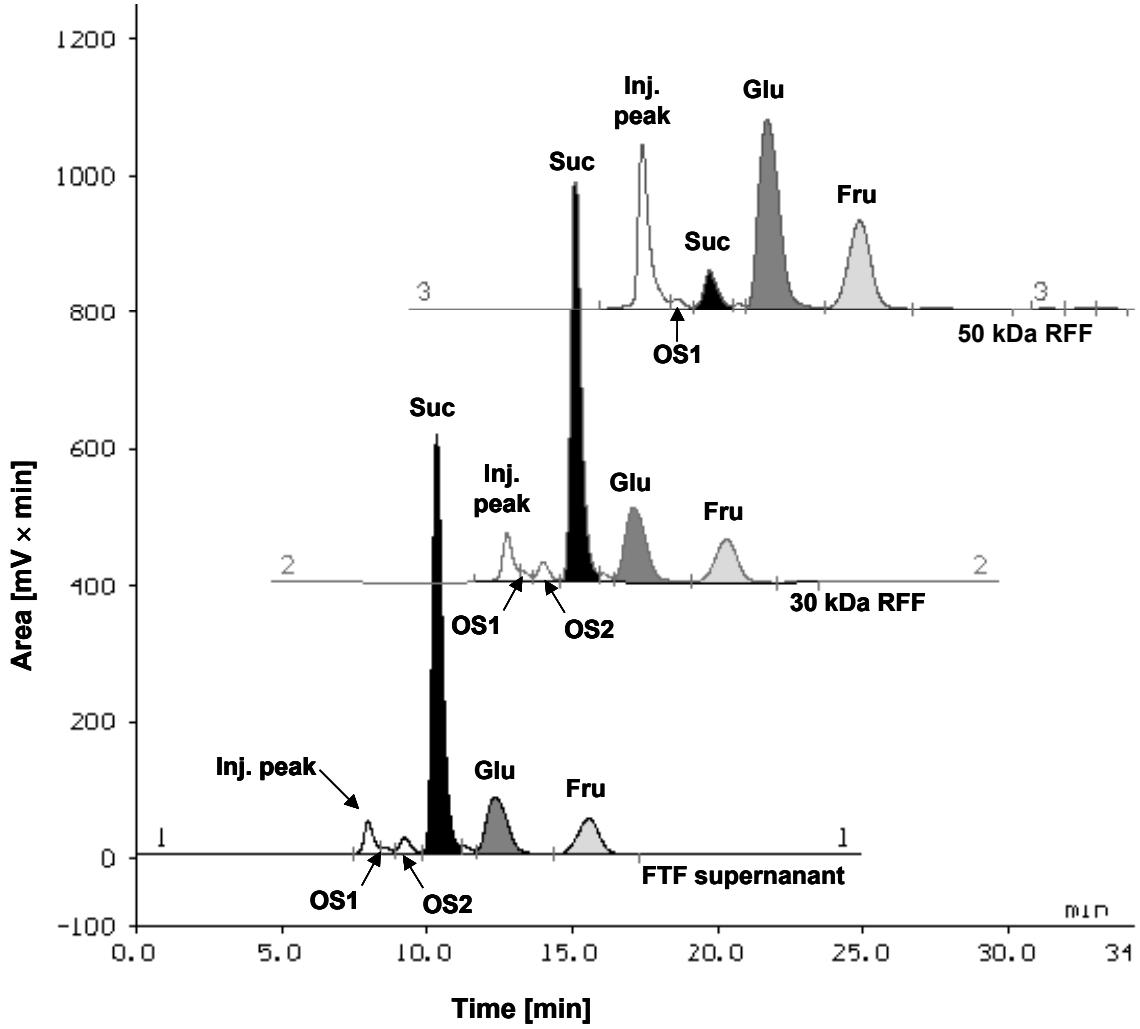


Fig. 36: IC chromatogram of sucrose hydrolysis reaction series with FTF after 24 h

By the chemical or enzymatic hydrolysis of sucrose, fructose and glucose are released in equimolar amounts. The molar balance between the glucose and fructose amount formed by sucrose hydrolysis with FTF, 30 kDa RFF and 50 kDa RFF shows lower values for fructose as for glucose. This occurs particularly in the reaction catalysed by the 50 kDa RFF, when the value for the fructose achieves only 40% of the glucose value. From this gap for fructose it becomes obvious that this monosaccharide can be consumed for oligomer synthesis. This affirmation is sustained also by the increase of the injection peak area that is evidently to notice in the chromatogram from Fig. 36 (a detailed explanation about the role of the injection peak is given at section 3.2.8). Consequently, the oligomeric compounds synthesised can be represented either by inulin or levan type oligosaccharides, respectively FOS.

As characteristic for FTFs and levansucrases with polymerising activities the oligomers formed start from the trimer 1-kestose, followed by the tetramer nystose, the pentamer fructosyl-nystose up to polymers. The polymerisation degree of these polymers can be up to 22 as reported for the levan synthesised by the levansucrase from *B. subtilis var saccharolyticus*) [63].

By comparing the R_f of the oligomers detected by TLC to those of the standards stachyose, raffinose and melibiose it can be supposed that the highest oligomer formed may be represented by a tetramer. As reported in literature for this FTF, the degree of polymerisation could be in the range of 5-7, the largest acceptor molecule that can react with the FTF from *B. subtilis* NCIMB 11871 being a pentasaccharide [24].

With regard to the mechamismus of the hydrolysis/polymerisation reaction catalysed by FTFs, kinetical and chemical studies of a related levansucrase from *B. subtilis* suggest a ping-pong mechanismus. According to this mechanism, each fructosyl unit is added one at a time onto an acceptor molecule and, since the initial acceptor is sucrose, the growing polymer chain acts subsequently as an acceptor [60].

Le Gorrec et al. reported the identification of three enzymatic activities in the FTF supernatant produced by *B. subtilis* NCIMB 11871 in the reaction with sucrose as substrate. The first activity of FTF is responsible for the transfructosylation reaction, which occurs in the presence of an acceptor. Secondly, the FTF can act as sucrase catalysing the hydrolysis of the substrates sucrose, raffinose and stachyose. Thirdly, the FTF can play the role of polymerase synthesising fructose polymers (FOS). For the reason that all methods tested to separate them failed, they suggest that these activities are catalysed by the same enzyme represented by a levansucrase and not by a FTF [59].

Previous papers reported that by the characterisation of the levansucrase from *B. subtilis var. saccharolyticus* the hydrolysis of sucrose is favoured at higher temperature, for example 37°C, whereas the levan formation occurs more effectively at low temperatures (4°C) and lower pH [57], [64]. The sucrose hydrolysis with FTF was investigated in this work at the optimal parameters for the transfructosylation reaction that means pH 6 and 50°C. Therefore, if of scientific interest for a future study regarding the hydrolysis catalysed by this FTF the optimal reaction conditions should be firstly determined.

The phytogenic tree of sucrose-hydrolysing enzymes suggests that they can be classified in two groups: one comprising fructosidases, catalysing sucrose hydrolysis (for example sucrase, invertase and levanase) and the other encompassing FTFs and levansucrases, catalysing transfructosylation from sucrose. It was reported in the literature that the examination of the homology between the primary structure of these enzymes revealed

five well-conserved regions in levansucrases and FTFs, but the specific functions of the catalytic residues within them are not fully understood [57].

A so called “sucrose box”, which is highly conserved in the streptococcal FTFs and levansucrases of bacilli appears to play a key role in sucrose binding. Therefore it has been earlier reported that the molecular basis of the first step of the catalytic process, the formation of a fructosyl-enzyme covalent intermediate appears to have been highly conserved [62]. Detailed research support the hypothesis that the FTFs of different sources may have a common origin or precursor, but the catalytic specificity of these enzymes could have diverged and adapted to ensure the needs of different species dependent on their ecological niche [60].

As transfructosylation by levansucrase is initiated by the cleavage of the sucrose β-(1-2)-fructosyl bond, it was assumed that the enzyme possesses an active center similar to those of glycosylhydrolases and glycosyltransferases [57], [61]. Recently, a crystal structure of *B. subtilis* levansucrase in its ligand-free and sucrose-bond have been determined and highlights a clear relationship between glycosidase families 68 and 43, suggesting that these enzyme families have developed from a common ancestor [65]. Still, the relationship between the structure and function of FTFs and levansucrases is only poorly understood.

3.2.10. Electrophoresis and isoelectrical focussing

One major protein band at about 33 kDa and some diffused protein bands (Fig. 37 A) as summarised in Tab. 17 were observed after the FTF supernatants were subjected to SDS-PAGE.

After isoelectric focussing (Fig. 37 B), several main protein spots with an approximately molecular weight of 40 kDa appeared at isoelectrical point (pI) values of 5.2, 5.3, 5.6, 5.8, 6.7 and 7.0. Besides, few undersized spots were identified at 27 kDa (pI 5.5), 70 kDa (pI 6.3 and 6.5) and 75 kDa (pI 6.1). All spotted proteins present an isoelectrical point situated in an acid-neutral range, so they are anions in neutral medium.

The N-terminal sequencing of all resultant protein bands by SDS-PAGE electrophoresis, led to the identification of the 33 kDa protein. Its sequencing revealed that this 33 kDa protein is represented by flagellin. According to NiceProt View of SWISS-PROT database, this 304 amino acids protein (entry name: FLA_BACSU; primary accession number: P02968) belongs to the bacterial flagellin family and is the subunit protein which polymerises to form the filaments of bacterial flagella.

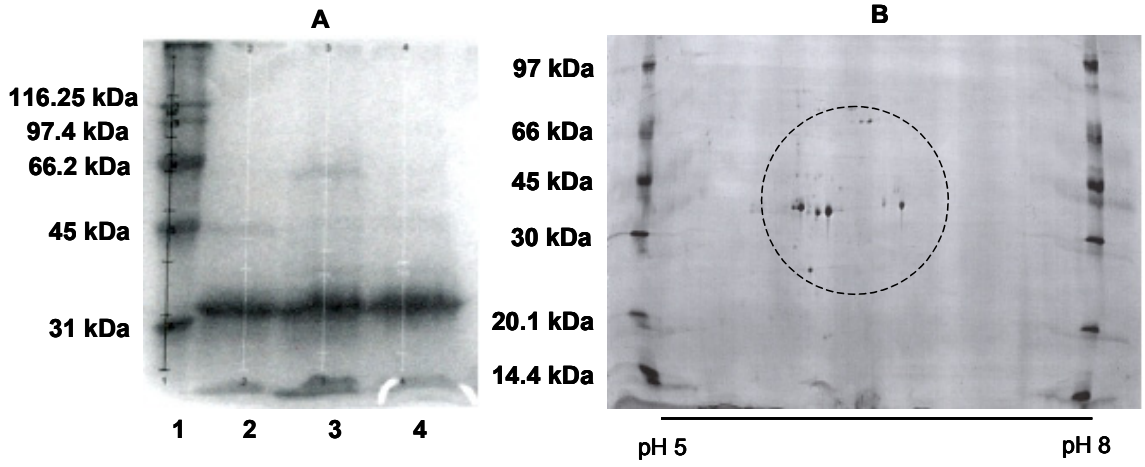


Fig. 37: A) Silver stained SDS-PAGE of FTF supernatants. Lanes: 1-protein marker; 2-culture supernatant (fermentation 1); 3, 4-culture supernatant (fermentation 2)
B) 2D-SDS gel: first dimension (horizontal)- pI determination; second dimension (vertical)-molecular weight determination.

Tab. 17: Results of SDS-PAGE of the FTF supernatants

Lane nr.	Molecular weight [kDa]			
Lane 2	32.98	44.92	-	-
Lane 3	33.40	38.33	62.98	71.18
Lane 4	33.51	38.44	40.41	165.82

For few protein bands similarities to several proteins from different microorganisms were found as summarised in Tab. 18.

However, the identity values are very low. For the rest of the protein bands, in the searched databases (EMBL and SWISS-PROT) no similarity was determined. Up to now, more than 19 bacterial FTFs have been reported in GenBank, but the results indicate that for any of the proteins no match to any FTFs or levansucrases was found.

Since the protein analysis methods were performed with the raw FTF supernatants, which contain also other extracellular proteins from *B. subtilis* and due to the fact that the FTFs diverge in characteristics (subject discussed comprehensively in section 3.2.8), no statement with regard to the molecular weight and pI of the studied FTF can be made.

Tab. 18: Protein database research report

Molecular weight [kDa]	Protein information	pI	Similarity [%]	Identity [%]
27.00	Response regulator SaeR *Staphylococcus aureus*	5.2	63	28
31.29	Transcriptional regulators *Corynebacterium glutamicum*	4.1	60	23
49.89	Dihydrolipoamide dehydrogenase E3 subunit of both pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes *Bacillus subtilis 33.40*	5.0	96	27
105.85	Similar to unknown proteins *Listeria innocua*	5.6	57	9

In order to characterise the FTF implied in this work in future studies, an essential step is a directed purification of this FTF by conventional methods such as salting-out, ultrafiltration or different types of chromatography, that should enable the separation of the proteins present in the FTF raw supernatant. In the presented work, ammonium sulphate precipitation was tested as described by *Cheetam et al*. [24], but this salting-out method led did not succeed in a purification of FTF (therefore was not described). According to the results discussed at section 3.2.8, ultrafiltration may represent an effective separation and concentrating method and consequently membranes with MWCOs higher than 50 kDa should be tested. The fractions resulted should afterwards be subjected both to protein analysis techniques and to transfructosylation, hydrolysis and polymerisation assays. The performing of these three reactions with the purified fractions should allow to verify if the FTF is the only responsible for these activities or if there are several enzymes involved (as described in section 3.2.9).

3.2.11. Purification of fructosyltransferase with ion exchange resins

A valuable information for performing ion exchange chromatography of proteins is the knowledge of the pI of the enzyme/protein of interest. Determination of the isoelectric point of FTF, as described in section 3.2.10, led to values in a range between 5.2 and 7 for nine main spots. For that reason, the adsorption tests with all ion exchangers were performed with the 30 kDa RTaF at the pH values of 5, 6 and 8.1. For the FTF studied in this work *Cheetam et al.* previously reported its successful purification by using DEAE-52 cellulose subsequent to the ammonium sulphate precipitation [24].

Anionic type exchange resins as DEAE sepharose and DEAE-52 celullose present positively charged groups (NH_3^+), whereas cationic types as CM Sepharose present negatively charged groups (COO^-). The proteins respectively enzymes, which possess both carboxylic and amino residues, can be adsorbed onto the exchange resin by reversibly binding to the opposite charged group. The elution occurs by increasing the ionic strength of the elution buffer system.

The 30 kDa RTaF gave one major band at 55 kDa, whereas the supernatants from the adsorption series exhibited diffuse protein bands also at 37 and 70 kDa as the SDS-PAGE results summarised in Tab. 19 show. The adsorption was succesfull at pH 5 and 6, while at pH 8.1 no spot was detected. It may be supposed that at this high pH the proteins might be denaturated, consequently agglomerated and sedimented by centrifugation.

Tab. 19: FTF purification with ion exchange resins

	CM sepharose			**DEAE-52 cellulose**			**DEAE sepharose**		
pH	5	6	8.1	5	6	8.1	5	6	8.1
Supernatant									
70 kDa							+	++	
55 kDa	++	++		++	++		+++	+++	
37 kDa				+				+	
Bond proteins									
70 kDa									
55 kDa	+	+							
37 kDa	+	+							

Furthermore, the black pigment present in the 30 kDa RTaF (for details see section 3.2.2) was adsorbed onto all the adsorbers, however stronger onto the DEAE-52 cellulose since this ion exchange resin exhibited the most intensiv black colour from all.

In view of the fact that the protein content of the 30 kDa RTaF was low, of about 0.06 mg/mL, the protein concentration of all centrifugates was noticeably diminished. Therefore no quantitative protein determination was achievable (the volumes of the samples were also not sufficient for further concentrating). For this reason the results are only schematically reviewed in Tab. 19. According to these results, it may be assumed that several protein bands could not be detected, such as those of approx. 40, 63 and 166 kDa which were identified by SDS-PAGE with concentrated samples of the FTF supernatants

(section 3.2.10). Based on these results it seems that at least some of the proteins of 37 and 55 kDa bind to the weak cation exchange resin CM sepharose, but it is difficult to come to an obvious conclusion.

It has to be underlined that the aim of this work was the enzymatic synthesis of the sucrose analogue Gal-Fru. Therefore, the research was first and foremost focussed on this topic. The concentration of FTF by ultrafiltration, the sucrose hydrolysis reaction, the electrophoresis, isoelectrical point determination and purification of FTF with ion exchange resins were performed supplementary, in order to acquire more information about the characteristics, properties and behaviour of the FTF.

3.2.12. Immobilisation of fructosyltransferase

It is well known that for technical applications the catalysts, particularly the enzymes should exhibit high activity and effectiveness. By immobilising, besides these two features the heterogeneous catalyst system ensures mechanical and operational stability, repeated use, thus economic and ecological advantages. Therefore, with regard to the efficiency of the Gal-Fru synthesis the immobilisation of the FTF was tested.

Immobilisation of several FTFs was reported in the literature and accordingly, the adsorption on different carrier materials such as anion exchange resins (DEAE 52 cellulose) [24], diatomaceus earth [66], chitosan derivative [67] and hydroxyapathite [59] seems to be the main immobilisation procedure. As described previously by *Rathbone et al.*, the studied FTF was successfully immobilised by adsorption onto DEAE 52 cellulose. The resultant biocatalyst showed an activity of about 80% as compared to the free enzyme, its activity being calculated from the formation of Xyl-Fru (xylsucrose) [44].

For this study, two methods were used for the immobilisation of FTF: binding to glutaraldehyde activated Trisopor® and direct covalent binding on Eupergit®.

Trisopor®-Amino porous glass beads (97% SiO_2) present a functionalised surface with amino groups [48].

Eupergit® represents a carrier material consisting of macroporous beads of acrylic polymers, which is used for covalent binding of enzymes of industrial potential for the production of fine chemicals and pharmaceuticals. Eupergit® is a copolymer of methacrylamide, N,N'-methylene-bis-methacrylamide and monomers containing oxirane groups. The chemical composition of Eupergit®C and Eupergit®C 250 L is identical, differing only by their content of oxirane groups (at least 0.93% and at least 0.36% respectively) and the porosity (bulk density of 0.6 g/mL and 0.3 g/mL respectively) [49].

From the immobilisation data, the ratio wet weight biocatalyst/dry weight biocatalyst (g/g) was calculated and gave ratios of approx. 3.14 for Trisopor®-Amino, 5.9 for Eupergit® C and 6.1 for Eupergit® C 250 L.

The three support materials were tested against a fixed amount of protein as in section 2.18 described. The immobilisation results summarised in Tab. 20 reveal that the Eupergit® C 250 L and Trisopor®-Amino immobilisates exhibits comparable high relative activities.

Tab. 20: Immobilisation of FTF

Biocatalyst	FTF activity [U/L]	Rel. activity [%]	Protein content [mg]	Protein content [%]	FTF activity/g dry weight biocatalyst [U/g]
30 kDa RTaF	267	100	0.71	100	-
Trisopor®-Amino	325	122	0.05	7	7.2
Eupergit®C	234	88	0.13	18	3.1
Eupergit®C 250 L	338	127	0.23	32	5.1

The different behaviour of the two types of Eupergit®, may be explained with the higher porosity and lower content of active oxirane groups in Eupergit®C 250 L (> 250 µmol/g dry weight) compared with Eupergit®C (> 600 µmol g/dry weight) [68]. The higher content of the oxirane groups of Eupergit®C compared to Eupergit®C 250 L result in a formation of multiple covalent linkages between the polymer and enzyme, which may disturb the globular structure of the protein and hence the activity.

The higher activities of the two immobilisates (adsorbed onto Trisopor®-Amino and Eupergit® C 250L) in comparison to the free 30 kDa RTaF as reference, led to the conclusion that the immobilisation can be seen as a further purification step for the enzyme (most of the disturbing compounds are probably not immobilised and removed in the washing step). The immobilisation of the FTF is overproportional with respect to total protein content.

Even the immobilisation on Eupergit®C can be considered very effectively, (despite the 12 % relative activity loss calculated), since *Rathbone et al*. succed to immobilise this FTF onto DEAE 52 cellulose with an activity yield of 80%. Furthermore, a previous immobilisation attempt on Eupergit® directed to a relative activity of only 8%. This immobilisation was briefly reported in the literature for the levansucrase from *Z. mobilis* ATCC 10988 (relative activity was calculated by taking the native levansucrase as

reference) [69].

Due to the high similar activity yields of the two biocatalysts obtained by adsorption onto Trisopor®-Amino and Eupergit®C 250 L, in order to evaluate their feasibility the operational stability of activity was estimated. This was achieved by applying the same sample of immobilised enzyme to a second consecutive activity determination batch-cycle over a time period of 24 h at the same conditions of pH, temperature and substrate-acceptor concentration. Recovery and reuse of the immobilised enzyme was carried out by filtering the biocatalyst from the reaction mixture, followed by washing steps, before starting a new batch cycle.

The results showed that, already after the second cycle, for Trisopor®-Amino and also for Eupergit®C 250 L the activity loss was in the range of 60%, related to the initial activity. In the case of Eupergit®C 250 L, stabilisation of the biocatalyst could be achieved after enzyme immobilisation when the residual oxirane groups are substituted by reaction with Cleland's reagent (dithiothreitol) as reported by *Katchalski-Katzir et al.*. They affirmed that after enzyme immobilisation, the bulk of oxirane groups are still present and can be easily modified in order to achieve the best "surroundings" for the enzyme [68].

3.2.13. Gal-Fru separation

As in section 3.2.7 comprehensively explained, in the FTF substrate-acceptor reaction, approx. 55% Gal-Fru is formed and 45% sucrose remains not converted. Since sucrose and Gal-Fru present the same molecular weight and nearly an identical structure (isomers), that makes clear that their separation is obviously difficult. Therefore, in order to obtain a Gal-Fru with high purity on preparative scale, the removal of sucrose represents an absolute requirement prior to preparative chromatographic separation.

For this purpose, a two step separation procedure was developed and established: the first step is represented by the sucrose removal from the Gal-Fru substrate solution by enzymatic treatment with high specific enzymes. The second step is the preparative chromatographic separation of monosaccharides, Gal-Fru and polysaccharides of the obtained reaction mixture. The Gal-Fru substrate solution represents the end reaction solution (after 24 h) with inactivated FTF, which contains residual sucrose, galactose, Gal-Fru, glucose and fructose.

Firstly, the Gal-Fru substrate solution, which contained about 55% Gal-Fru, is subjected to a treatment, with immobilised dextransucrase from *Leuconostoc mesenteroides*/α-glucosidase or instead of this with free dextransucrase from *Streptococcus oralis* (for

details see section 2.20) in view to remove the sucrose.

The native dextransucrase (DS) from *Leuconostoc mesenteroides* NRRL B 512 F has a K_M value for sucrose of 29.4 mmol/L, about 30 times higher as the DS from *Streptococcus oralis* (0.9 mol/L). The high K_M value of DS from *L. mesenteroides* imposes the combined two steps enzymatic treatment with α-glucosidase. For this purpose and with regard to the high cost of the α-glucosidase, the Gal-Fru substrate solution is treated at first with immobilised DS and once the sucrose concentration achieves a value below the K_M of DS, α-glucosidase, which has a very low K_M, is added until sucrose is not detected anymore as depicted in Fig. 38.

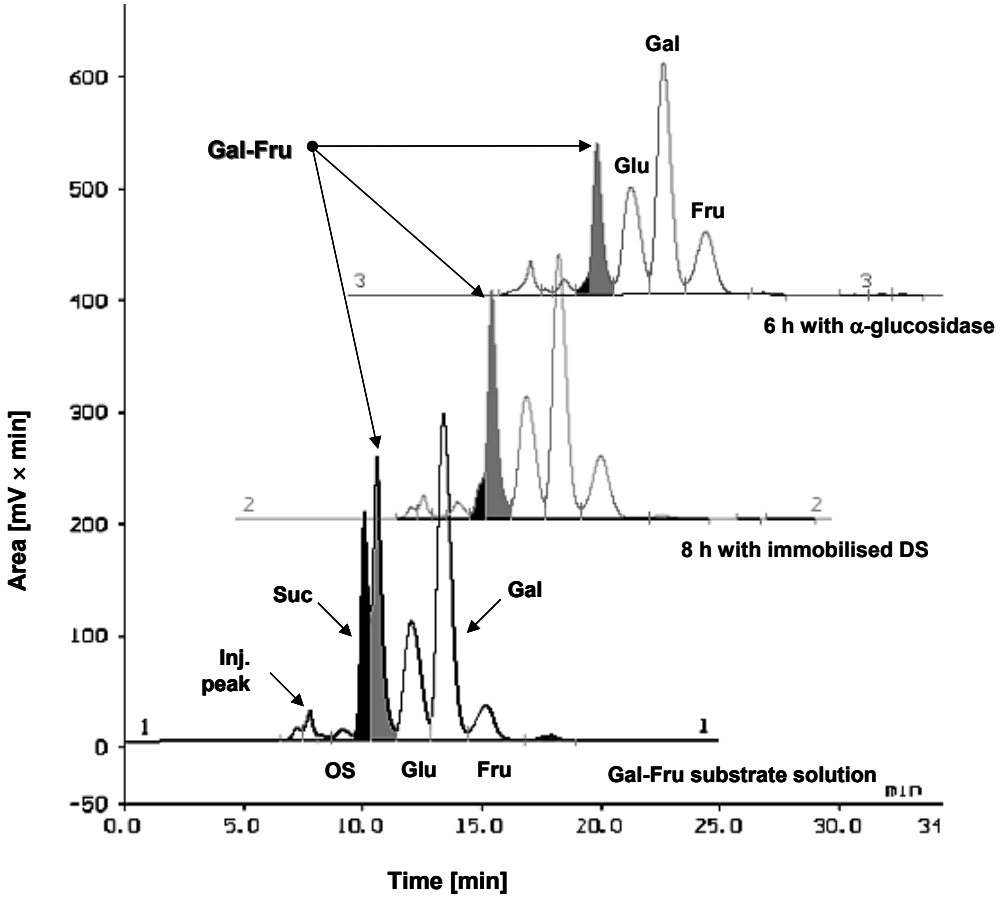


Fig. 38: Treatment of the Gal-Fru substrate solution with immobilised DS/α-glucosidase

The elimination of sucrose on the second pathway with wild type DS from *Streptococcus oralis* cloned in *E. coli* represents an alternative low-cost one step pathway (in the frames of this joint project, since the recombinant DS was provided by a collaborating working group), that ensures complete degradation of sucrose in only one reaction. An example is

illustrated in Fig. 39. Since the recombinant DS was recently discovered and its behaviour had firstly to be studied, the first attempts to remove sucrose were performed with the system immobilised DS/α-glucosidase.

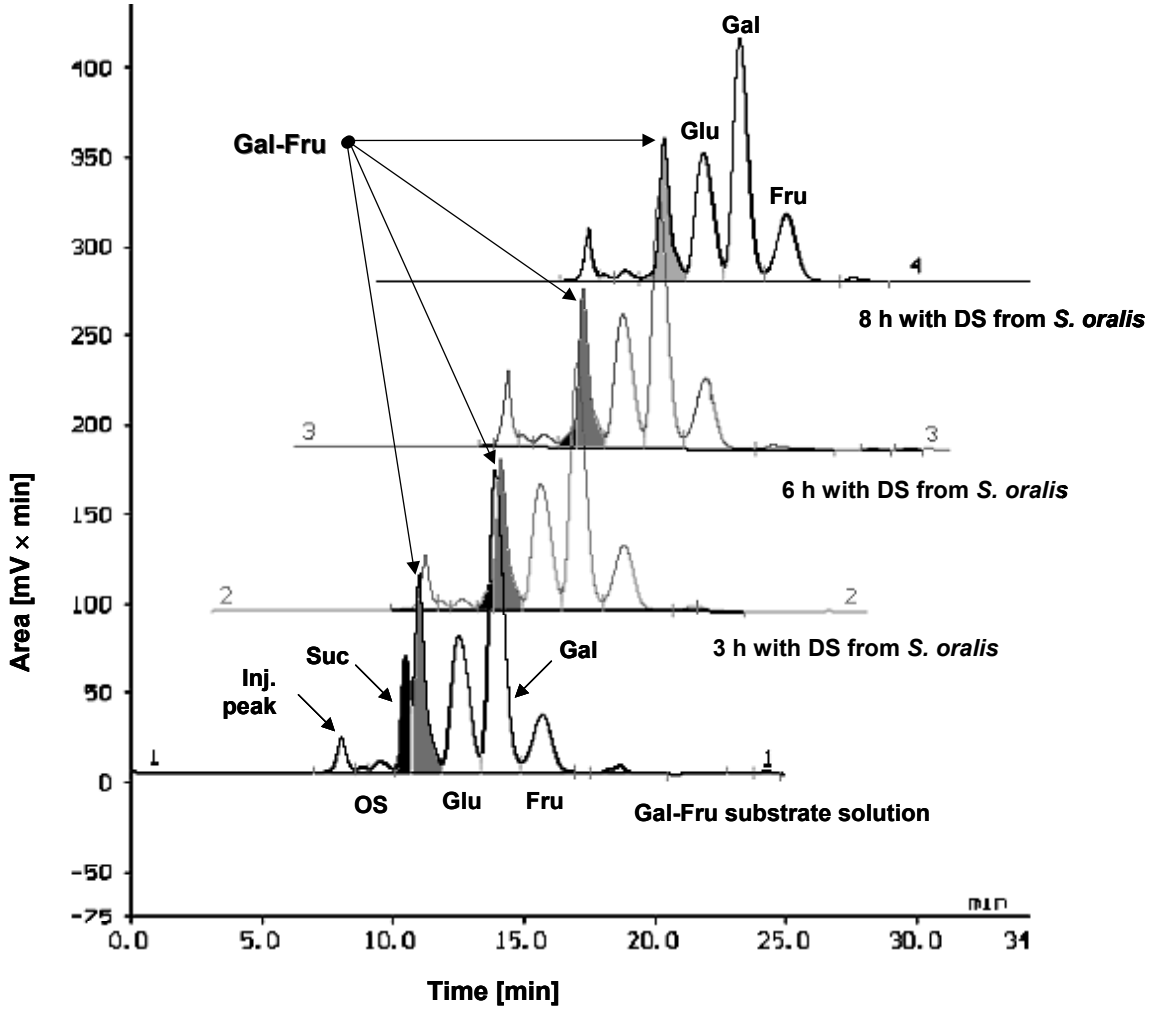


Fig. 39: IC chromatogram of the Gal-Fru substrate solution treatment with free DS from *S.oralis*

DS (EC 2.4.1.5) catalyses two kinds of reaction: the primary reaction is the synthesis of dextran from sucrose and the secondary reaction is the transfer of D-glucose from sucrose to carbohydrate acceptors, that are added [32]. As it can be seen on the TLC plate shown in Fig. 40, DS attacks sucrose leading to fructose and higher glucosyl-oligomers up to dextran. After 8 h reaction, sucrose is completely degraded and the spot corresponding to Gal-Fru begins to turn pale.

The new spots, detected on the TLC plate, are dextran and oligosaccharides (OS), synthesised by DS with sucrose and acceptors fructose, glucose, galactose and probably even Gal-Fru present in Gal-Fru substrate solution. The decrease of the Gal-Fru concentration could be also explained by hydrolysis by DS, which leads to galactose and fructose.

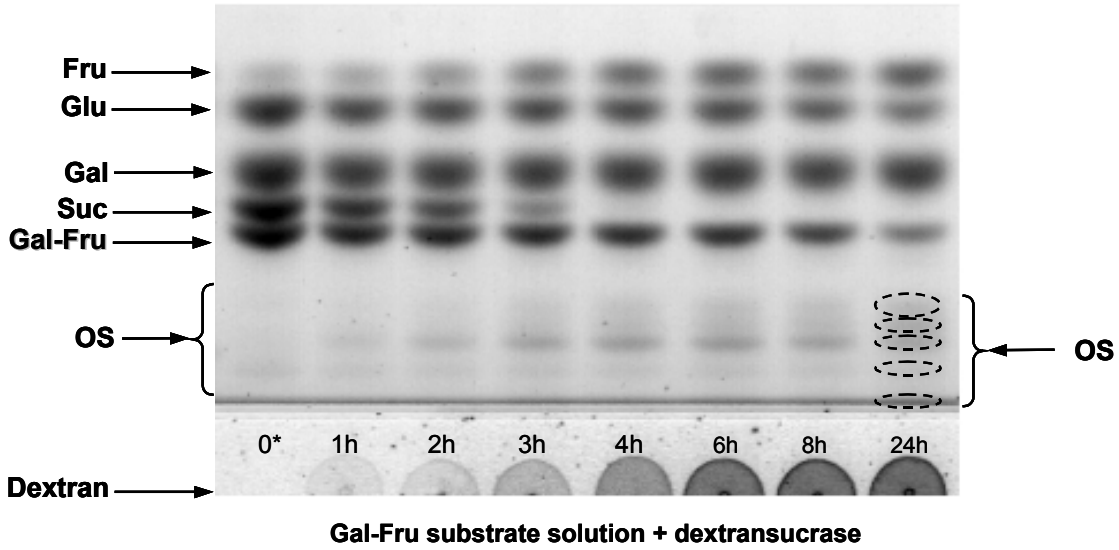


Fig. 40: Gal-Fru structure determination with DS from *S.oralis* (*Time 0 represents the start of the DS reaction with the Gal-Fru substrate solution)

Sucrose is the only natural substrate for DS, but several sucrose analogues, such as Gal-Fru and Xyl-Fru (xylsucrose) [28] and sucrose derivatives modified at C-3 and C-6, have been studied as acceptors, inhibitors and substrates for the GTF of *Streptococcus mutans* 6715 [70], [71].

Neither the two sucrose analogues and none of the C-3 modified sucrose analogues, tested by *Binder&Robyt*, were found to be substrates, although two of them, 3-deoxysucrose and 3-fluorodeoxysucrose, showed acceptor properties. In the case of the C-6 sucrose derivatives, only thiosucrose acted as substrate, when acceptors were present, the rest acting only as different types of inhibitors. It must be mentioned that the DS reaction with Gal-Fru as substrate was poorly studied before [28], [70].

After the enzymatic step, the α-glucosidase or the DS from *S. oralis* is inactivated and the resulting mixture of Gal-Fru, mono- and oligosaccharides is subjected to preparative chromatographic separation.

Three types of stationary phases were tested for their suitability as chromatographic medium for the separation of Gal-Fru. The most successful preparative separation of the Gal-Fru from the FTF reaction mixture was achieved with PCR 6, a strong acid cation-exchange resin, having sodium as counter ion. It is applied industrially for the separation of glucose/fructose mixtures and the removal of residual sugar from molasses. The column was applied at 70°C, in order to exclude the formation of broad peaks or doublets due to the resolution of anomers and to accelerate the diffusion-controlled partitioning process. A major economic and environmental advantage is represented by the fact that water is the eluent used for the separation.

An example of the carbohydrate concentrations of the reaction mixtures subjected to the separation are shown in Tab. 21 and Tab. 22. The related chromatograms are shown at the Appendix section: Fig. 55 and Fig. 56.

Tab. 21: Start concentrations of the reaction mixture for preparative chromatography after sucrose removal with immobilised DS/α-glucosidase

Carbohydrate	Area [mV × min]	Concentration [g/L]
OS	26.4	0.5
Sucrose	2.6	10.7
Gal-Fru	40.4	166.2
Glucose	24.2	18.2
Galactose	56.6	43.9
Fructose	23.2	18.8
Total:	**173.2**	**258.3**

Tab. 22: Start concentrations of the reaction mixture for preparative chromatography after sucrose removal with DS from *S. oralis*

Carbohydrate	Area [mV × min]	Concentration [g/L]
OS	61.4	25.2
Raffinose	75.2	13.0
Gal-Fru	377.1	98.8
Glucose	217.4	51.6
Galactose	444.1	104.6
Fructose	208.8	46.3
Total:	**1 384.0**	**339.5**

It must be pointed out that the concentration of oligosaccharides and Gal-Fru are relative,

the data being calculated with the response factors of stachyose and of sucrose respectively (from IC calibration report).

As illustrated in the chromatogram presented in Fig. 41 and Fig. 42, the sugars are eluted in decreasing order of the molecular weight, showing that the size-exclusion mechanism is predominant. The oligosaccharides emerge firstly (after approx. 650 mL elution volume), followed by the disaccharides sucrose and Gal-Fru and, lastly, the monosaccharides glucose, galactose and fructose.

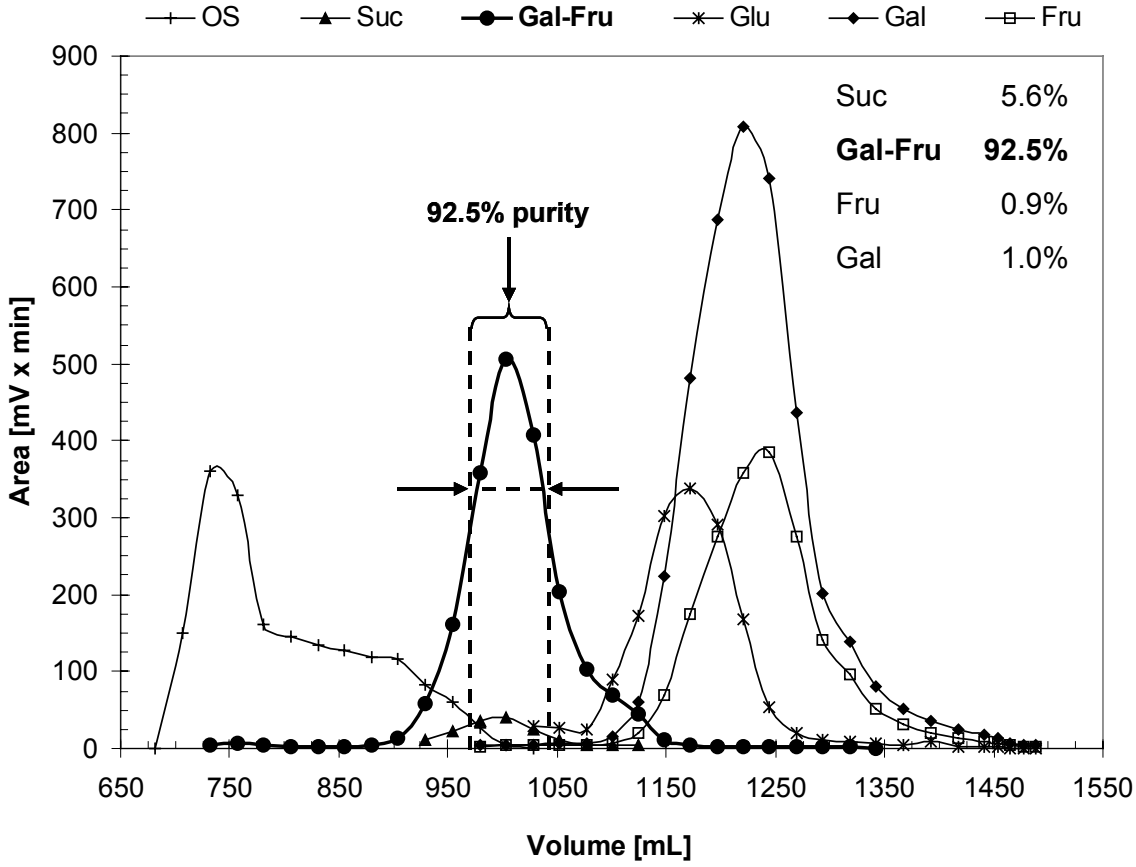


Fig. 41: Chromatogram of the Gal-Fru separation after enzymatic treatment with immobilised DS/α-glucosidase

The major part of Gal-Fru, contaminated with traces of sucrose, galactose and fructose, was found to be present from approx. 950 mL to 1080 mL eluent volume. The purest eluted fractions from 980 mL to 1025 mL eluent volume won on both sucrose removal pathways were pooled and concentrated by freeze-drying giving a product with a purity of 92.5% and 94.2% respectively, values obtained by IC analysis. As example, a chromatogram of the 92.5% Gal-Fru, resulted by the separation illustrated in Fig. 41 is

shown in Fig. 43.

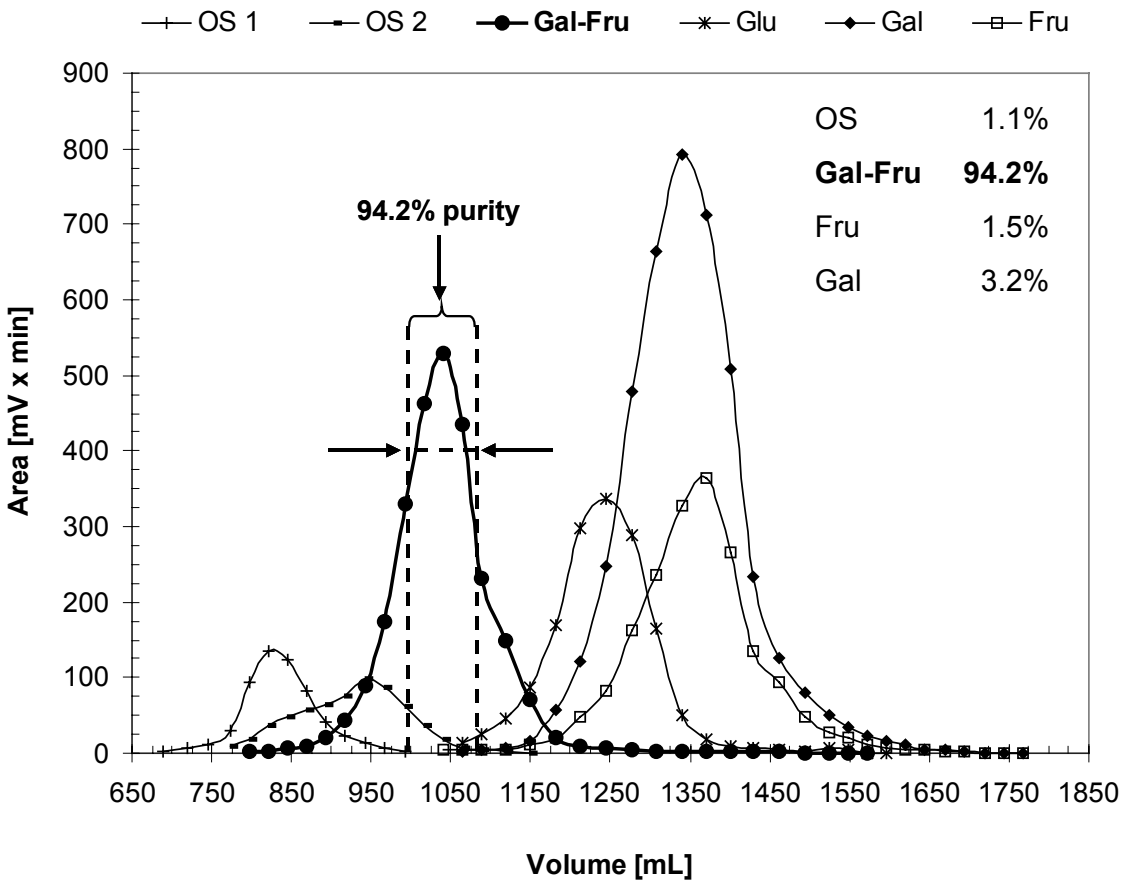


Fig. 42: Chromatogram of the Gal-Fru separation after enzymatic treatment with free DS from *S.oralis*

For a further purification and for an advantageous valorising of the product in order to minimise its loss, after the preparative chromatographic step, the fractions containing predominantly Gal-Fru from 60% up to 80% Gal-Fru, were pooled, evaporated under reduced pressure and subjected to a second chromatography by the same procedure. After freeze-drying of the collected purest fractions, a Gal-Fru with 95% purity was obtained.

The experimental data which represent the basis for the assembled chromatograms illustrated in Fig. 41 and Fig. 42 are summarised in Tab. 25 and Tab. 26 in the Appendix.

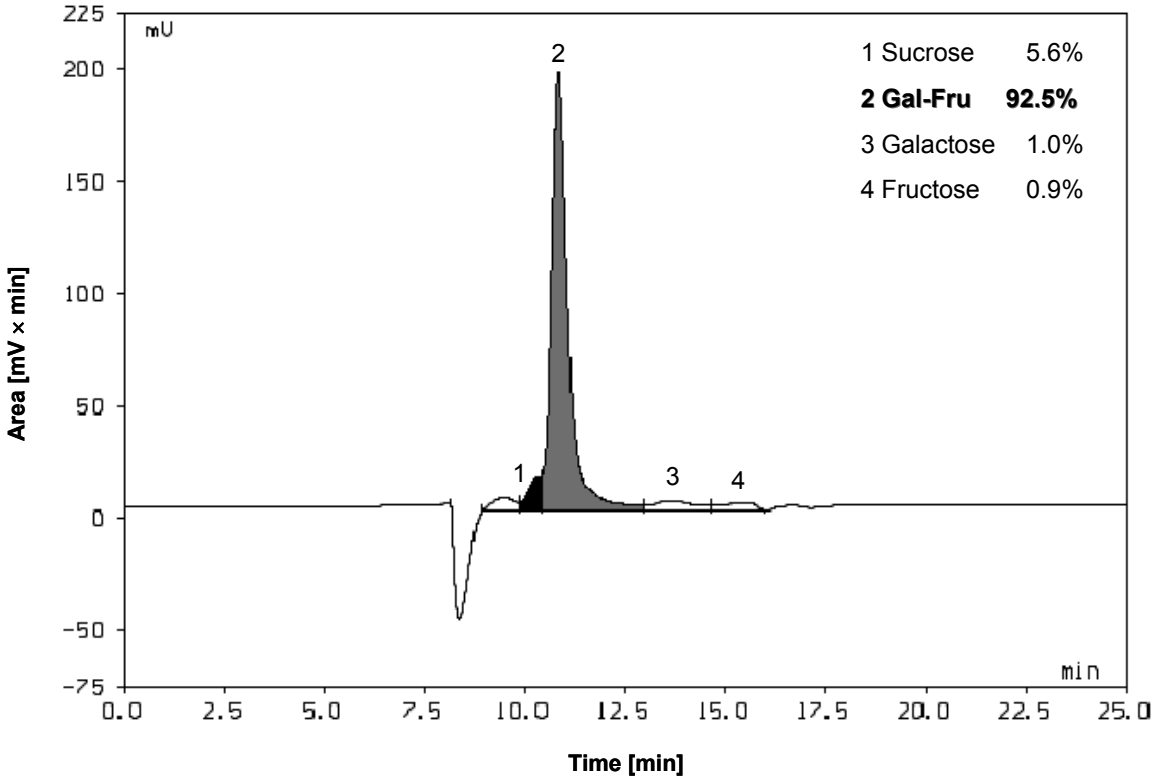


Fig. 43: IC chromatogram of the Gal-Fru from Fig. 41

3.2.14. Gal-Fru characterisation

It has to be emphasized that analysis of the structure and glycosidic bond of this disaccharide product were not treated in detail up to date. For this reason, accurate analysis for the Gal-Fru structure characterisation was performed with analytic (nuclear magnetic resonance spectroscopy-NMR) and enzymatic tools (invertase).

3.2.14.1. Enzymatic structure determination

Invertase was used as tool for the enzymatic Gal-Fru structure determination. For this purpose, tests were performed as well with the Gal-Fru substrate solution as also with the purified Gal-Fru.

Invertase hydrolysis the non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides as sucrose and saccharides of sucrose type, such as oligosaccharides from the raffinose series (see section 1.2, Fig. 6) and inulin [72].

By the hydrolysis of the Gal-Fru substrate solution with invertase, both disaccharides, sucrose and Gal-Fru, were degraded simultaneously, fructose, galactose and glucose

being the final reaction products, as shown in Fig. 44.

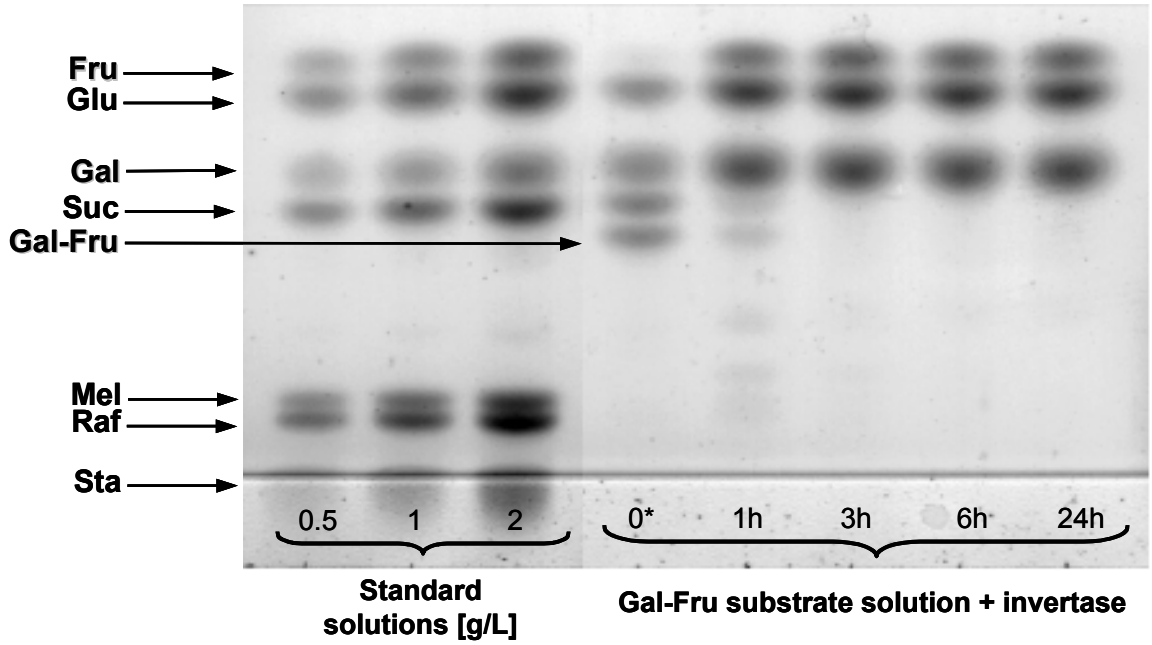


Fig. 44: Degradation of Gal-Fru by invertase (*Time 0 represents the start of the invertase reaction with the Gal-Fru substrate solution)

In order to obtain evident information about the product structure, a second test series was carried out with purified Gal-Fru. As shown in Fig. 45, the results indicate that the hydrolysis by yeast invertase leads to galactose and fructose in equimolar amounts (calculated from the IC analysis) as summarised in Tab. 23. This result confirms that the synthesised sucrose analogue is undeniably composed of galactose and fructose. The successful attack of β-fructofuranosidase on Gal-Fru indicates furthermore that this new product has the same structure as sucrose: the fructosyl residue has a β-furanosidic conformation and is bond to galactose by a β-2,1 linkage.

Tab. 23: Results of the Gal-Fru (85% purity) treatment with invertase

Carbohydrate	Concentration [mmol/L]		Balance
	Start (time 0)	End (after 6 h)	
Oligosaccharides	56.5	69.3	**12.8**
Gal-Fru	303.4	100.3	**203.1**
Galactose	22.4	194.8	**172.4**
Fructose	28.0	231.7	**203.7**

Nevertheless, after six hours reaction time a gap in the galactose concentration of 31.3 mmol/L galactose, equivalent to 5.6 g/L can be calculated (by comparing to the fructose concentration). This gap may be firstly due to experimental errors. Secondly, the oligosaccharides present in the 85% Gal-Fru preparation might react with the fructose released by invertase attack in order to form polymers. It has to be underlined that these oligosaccharides present in the Gal-Fru substrate solution are residual impurities, which remained after the Gal-Fru separation procedure (they were formed in the DS reaction as described in section 3.2.13).

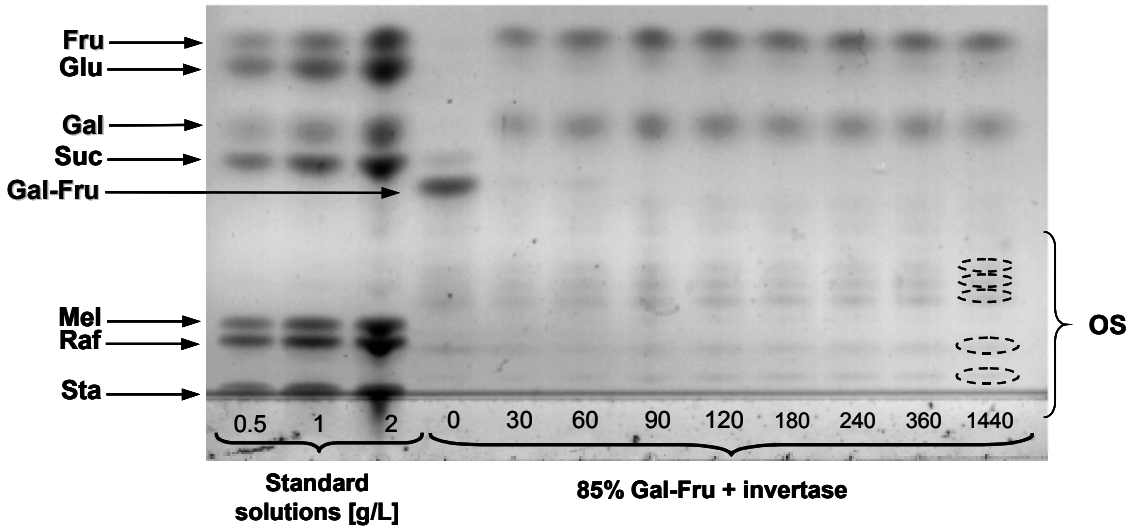


Fig. 45: Gal-Fru reaction with invertase

This second supposition is confirmed by the detection on the TLC plate shown in Fig. 45 of five spots under sucrose corresponding to oligosaccharides. It is well known that invertase exhibits also a transfructosylation activity when acting on high sucrose concentrations, producing as well FOS (for example the invertase obtained from *Aspergillus sp.*, *Penicillium sp.*, *Saccharomyces cerevisiae*) [25]. In the above presented invertase reaction it may be also assumed that instead of sucrose, Gal-Fru may act as substrate leading to the formation of FOS.

3.2.14.2. Analytical methods for Gal-Fru characterisation

Structural evidence for Gal-Fru was secured by the ^{1}H-NMR (Fig. 46) and ^{13}C-NMR (Fig. 47) spectroscopy. The elucidation of the new sucrose analogue complex structure was possible only by the combination of the data acquired from the ^{1}H-NMR, ^{13}C-NMR and 2D-

NMR spectra.

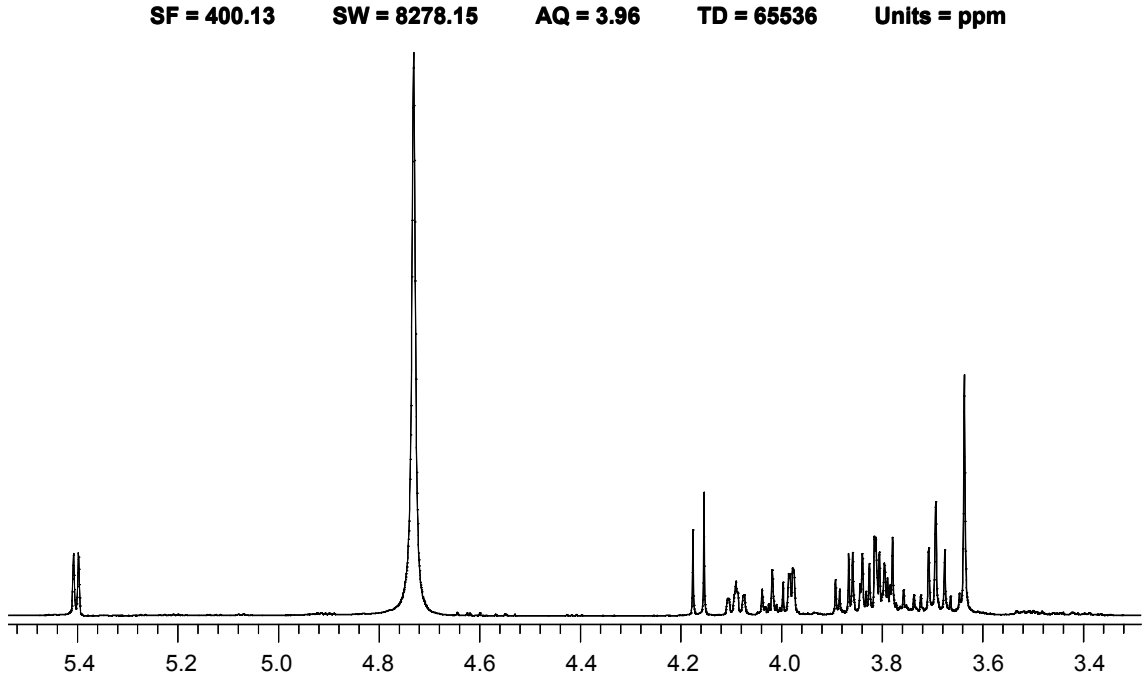


Fig. 46: ^{1}H-NMR spectrum of Gal- Fru

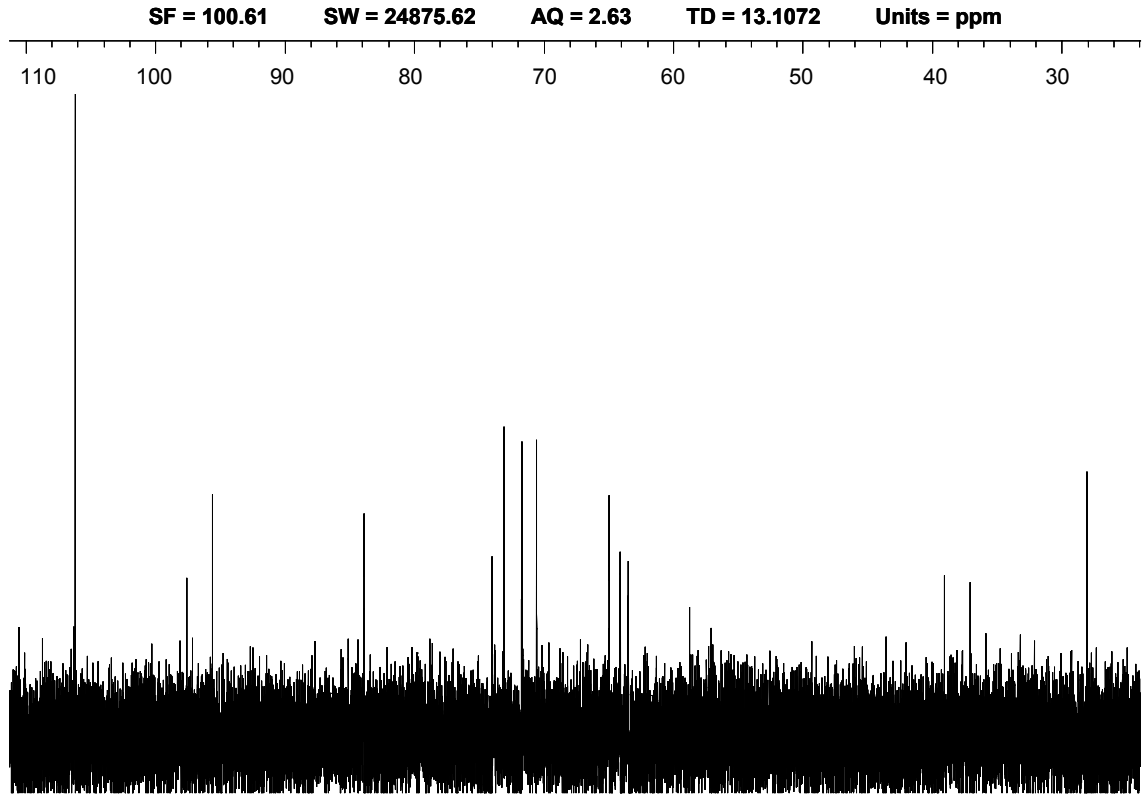


Fig. 47: ^{13}C-NMR spectrum of Gal-Fru

The duplet at δ 5.40 ppm exhibited the expected anomeric coupling constant $J_{1,2}$ of 3.9 Hz, characteristic for the anomeric proton of an α-(1-2)-glucosidic linkage. The main peaks in the ^{1}H-NMR spectrum were assigned using 2D-COSY spectroscopy. On this pathway, it was possible to measure most of the coupling constants. The values observed for the couplings of proton H-4 ($J_{3,4}$ 3.2, $J_{4,5}$ 0.9) suggested a galactopyranose residue. The complete interpretation of the ^{13}C-NMR spectrum was performed using 2D-^{1}H/^{13}C correlation spectroscopy (HMBC, HMQC).

According to these results, it can be concluded that the transfructosylation product is an O-β-D-fructofuranosyl-(2-1)-O-α-D-galactopyranoside having a fructosyl residue bond to galactose by a β-2,1-linkage.

For the accurate characterisation of the Gal-Fru spectra, the chemical shifts are summarised in Tab. 24 and expressed in δ values.

Tab. 24: ^{1}H- and ^{13}C-NMR data for Gal-Fru

Galactosyl-fructoside		
Carbon atom position	**δ_C**	**δ_H (*J*, Hz)**
1	94.87	5.40 (d, 3.9)
2	70.55	3.76-3.85 (m)
3	71.70	3.86-3.89(dd, 3.2, 10.5)
4	71.67	3.98-3.99 (dd, 0.9, 3.2)
5	73.99	4.07-4.11 (dt, 0.9, 6.4)
6	63.44	3.68-3.70 (t, 6.4)
1’	64.08	3.64 (s)
2’	106.20	-
3’	79.12	4.15-4.18 (d, 8.7)
4’	76.72	4.00-4.04 (t, 8.7)
5’	83.86	3.76-3.85 (m)
6’	64.93	3.76-3.85 (m)

The main chemical shifts of the IR spectrum (in KBr) shown in Fig. 48, were recorded as follows: ν_{max} 3428, 2945, 1132, 1087, 1049, 1017 cm^{-1}.

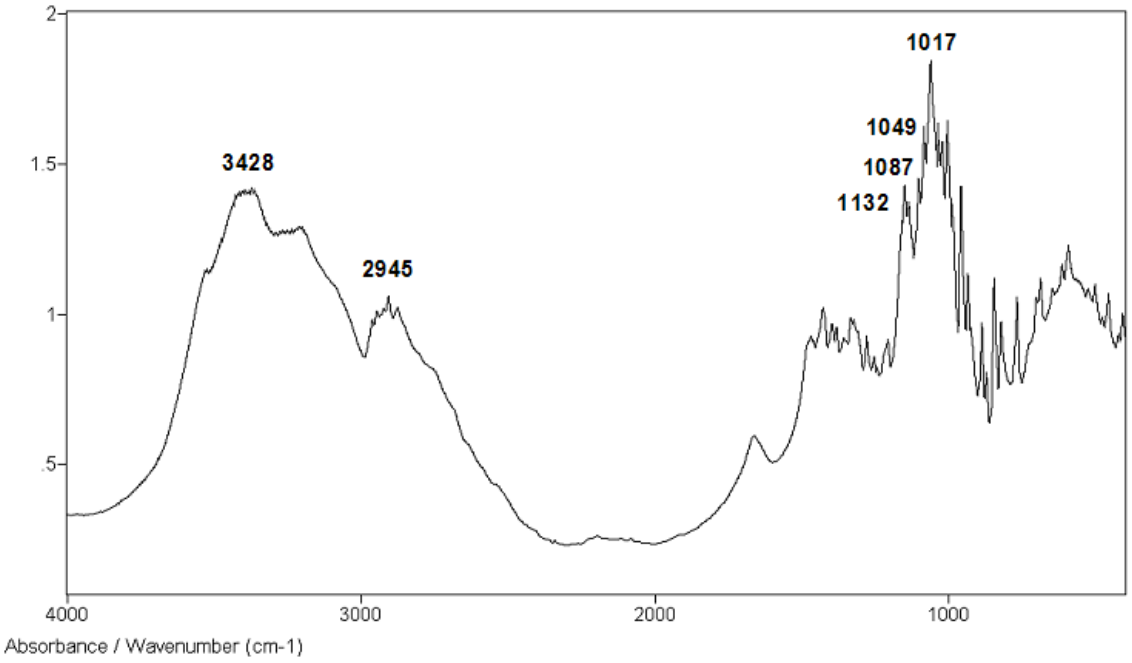


Fig. 48: FTIR spectrum of Gal-Fru

Galactosyl-fructoside characterisation: fine white solid (Fig. 49); mp 160-165°C (literature value 175°C, with sintering at 170°C) [28], [73]; $[\alpha]_D^{20}$ = +81.2 ± 0,5° (c 1, H_2O) [28], [72]; R_f 0.42 ± 0.03 (ethylacetate/ isopropanol/ water: 6/ 3/ 1); color reaction with N-(1-naphtyl)-ethylenediamine: violet-red.

Elementary analysis: Theoretic calculated: 42.07% C and 6.43% H. Found: 39.38% C and 6.82% H (due to the impurities).

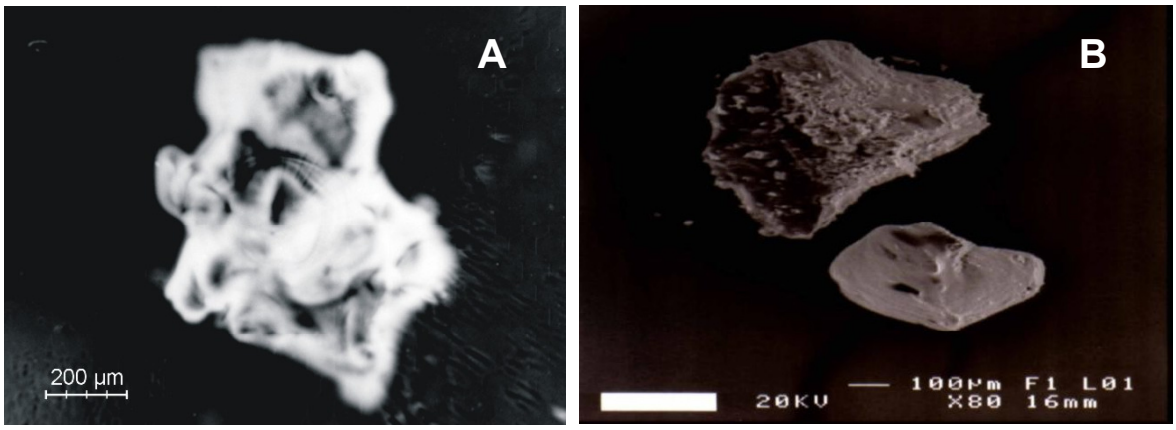


Fig. 49: Micrograph of freeze-dried Gal-Fru of 92.5% purity: A-microscopy; B-scanning electron microscopy

3.2.15. Discussion

The goal of this work was to synthesise the rare sucrose analogue galactosyl-fructoside starting from the renewable material sucrose. For the aimed objective, key topics of the research were: finding the appropriate biocatalyst, establishing a suitable separation method and characterisation of the product. Additional studies regarding the immobilisation and characterisation of the enzyme were also performed, in order to acquire an overview perspective.

The starting point of this work was the screening of several FTFs and levansucrases for their ability to synthesise Gal-Fru from sucrose in the presence of galactose. The only FTF that exhibited the required transfructosylation property was the extracellular FTF from *B. subtilis* NCIMB 11871. This exo-enzyme, classified as a monosaccharide specific FTF (EC 2.4.1.162), was produced from the non-pathogenic, gram-positive and GRAS status microorganism *B. subtilis*, by fermentation with 1% sucrose salt minimal medium [24].

In the framework of this thesis it was established that this enzyme with the systematic name of α-D-aldosyl-β-D-fructoside-aldose-1-β-D-fructosyltransferase catalyses the following three reactions:

a. Transfructosylation, the main FTF reaction represented by the transfer of the β-fructofuranosyl residue from donor substrates in which the leaving fructosyl group is bound to the anomeric carbon of an aldose (as sucrose, raffinose and stachyose) to the 1C of the acceptor galactopyranose, leading to the sucrose analogue Gal-Fru.

b. Hydrolysis of the substrate sucrose to the monosaccharides fructose and glucose

c. Polymerisation: synthesis of fructose polymers (FOS of inulin or levan type)

These results agree to those obtained by *Le Gorrec et al.*, which reported previously also the identification of these three enzymatic activities in the FTF supernatant produced by *B. subtilis* NCIMB 11871 [59].

The studies revealed that the transfructosylation catalysed by FTF with the substrate-acceptor system sucrose-galactose is an equilibrium reaction: it occured with maximal 55% Gal-Fru yield and correspondingly 55% sucrose conversion (after 24 h). The equilibrium establishes between the forward and reverse reaction, among the main reaction, the Gal-Fru synthesis and the sucrose formation, respectively.

This FTF may be suitable for commercial use in order to produce Gal-Fru, since the reactions are carried out at rather high concentrations of sucrose as substrate (40%). The use of the substrate sucrose represents furthermore a major economical advantage, when compared to the system raffinose-galactose, described before in the literature for the synthesis of Gal-Fru [28]. The ratio sucrose price/raffinose price is of approx. 1/105, therefore the benefit of performing the reaction with sucrose is evident. For industrial application, the reaction may be adapted and performed as well with technical sugar containing substrates, such as molasses. However, the influence of the impurities on the Gal-Fru synthesis should be firstly investigated.

In the presence of sucrose alone, FTF shows two secondary activities, sucrose hydrolysis and polymer synthesis. The calculation of the initial reaction rate led to low values indicating that with this FTF the sucrose hydrolysis occurs relatively slow compared to the transfructosylation.

The optimum pH and temperature of the enzyme were determined with regard to Gal-Fru synthesis. The maximal FTF activity for transfructosylation was achieved for a pH value of 6 and at 50°C, in agreement with the literature data. The high sucrose concentration and the elevated FTF optimal temperature can effectively prevent the infection of the reaction mixture. A high transfructosylation temperature is desirable also to surmount the viscosity of the high concentrated substrate-acceptor solution.

The determination of the activation energy yielded a value of 21.4 kJ/mol. It has to be underlined that this parameter is apparent, because it characterises a non-purified enzyme, a fact that may give an explanation about its small value in comparison to the majority of pure enzymes.

In view of a cost-effective use and a potential industrial application, the FTF was immobilised on three carrier materials. Comparable high activity yields were achieved with the Eupergit® C 250 L and Trisopor®-Amino immobilisates. Therefore and furthermore with consideration to carrier material cost, Eupergit® C 250 L was chosen to be the suitable matrix and consequently was also tested with concern to the operational stability. In most cases, by immobilising of lower purity intra- and extracellular enzymes (approx. 10% of theoretical maximal purity) will result a catalyst more stable than obtained with highly purified enzymes [49]. For the studied biocatalyst the immobilisation led to a decrease of activity yield in the second cycle, the biocatalyst maintaining only about 40% of its initial activity. The optimisation of the immobilisation procedure (pH and temperature value, ionic strength of the binding buffer, immobilisation time and ratio of protein to the matrix) may lead to the increase of the operational stability. The resultant biocatalyst may be appropriate for innovative purposes, such as the synthesis of novel oligosaccharides in a

fixed, as described for the “flow-through” synthesis of Xyl-Fru [44] or fluidised bed reactor.

The fact that sucrose and Gal-Fru have the same molecular weight and nearly an identical structure (isomers) makes clear the difficulty regarding their separation. In order to surmount this issue sucrose must be removed, prior to preparative chromatographic separation, in view to obtain a Gal-Fru with high purity. Accordingly, a two step separation lab-scale procedure was established for the isolation of a high purity product. In the first enzymatic step, sucrose is removed either by the attack of the system immobilised DS from *L. mesenteroides*/α-glucosidase or by the native DS from *S. oralis*. Afterwards, in the second step, the reaction mixture is subjected to preparative chromatographic separation. By performing this two step separation method, Gal-Fru with at least 92.5% purity can be obtained. For large-scale purposes, the cation exchange chromatographic system can easily be scaled-up without any risk providing both economical and environmental advantages.

By treatment with yeast invertase Gal-Fru was hydrolysed totally to monosaccharides galactose and fructose. Its susceptibility to hydrolysis by invertase confirmed that the fructosyl residue has a β-furanoside conformation. That the galactosyl residue has an α configuration was validated by measuring a positive optical rotation for this disaccharide.

On the basis of enzymatic behaviour (by invertase attack) and optical activity, NMR and FTIR spectral data, it was demonstrated that the product synthesised is an O-β-D-fructofuranosyl-(2-1)-O-α-D-galactopyranoside.

The studies performed for the determination of the molecular weight and isoelectric point (pI), as also the concentration by ultrafiltration and purification by adsorption on ion exchange resins of the FTF led to no evident conclusions, thus they should be regarded as basis pre-experiments for future research.

Summarising, the entire process as developed and established in this work, from the bacterial strain to the final product, Gal-Fru involves several steps as presented in Fig. 50. In the first step the *B. subtilis* strain has to be cultured for the FTF production. The extracellular FTF accumulates in the culture medium and the fermentation has to be stopped, when the maximum FTF activity is achieved. The separation of enzyme from cells can then succeed by centrifugation. The transfructosylation reaction can be started by adding the FTF supernatant to the substrate-acceptor system (sucrose-galactose) solution. After inactivation of the enzyme, the reaction mixture has to be subjected to a two steps separation procedure that ensures the isolation of a high-purity product.

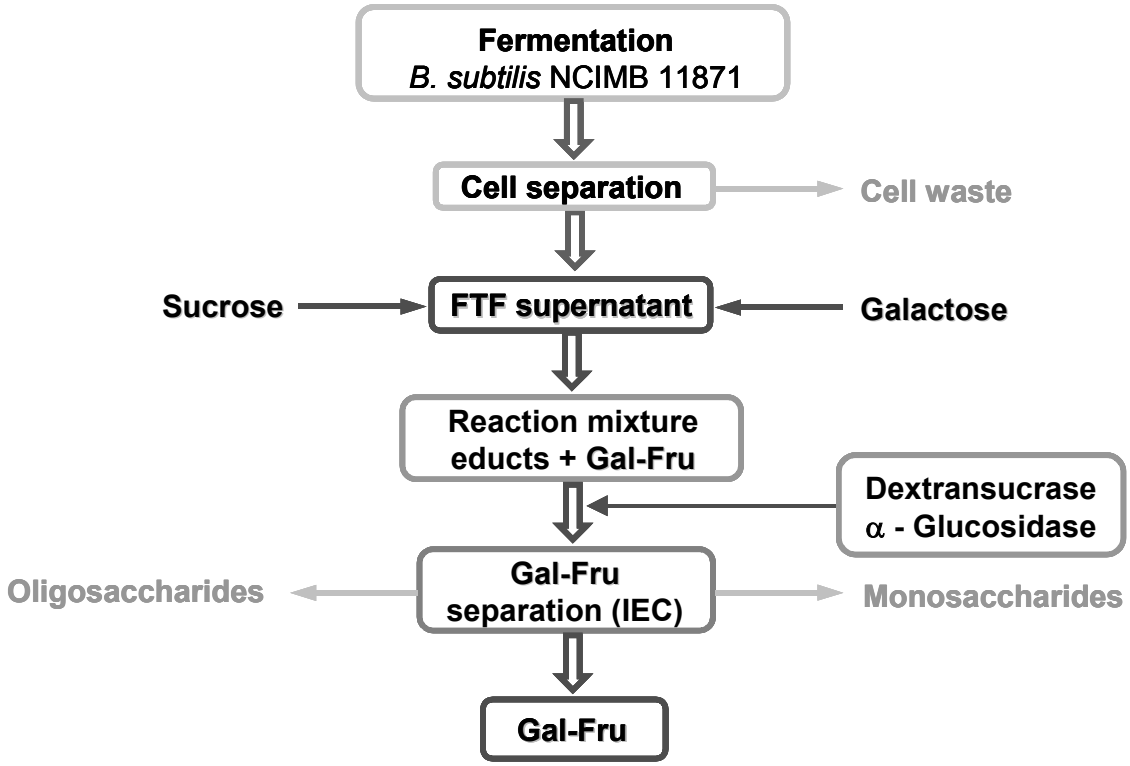


Fig. 50: Multi-step process for the production of sucrose analogue galactosyl-fructoside

3.3. Summary

The development of sugar industry particularly focussed to exploit renewable materials and glycobiotechnology field is leading to new products such as oligo-, polysaccharides and glycoconjugates that are alternative sweeteners, valuable cosmetic ingredients, potential pharmaceuticals and components in drug design. Moreover, carbohydrates and glycoconjugates are gaining importance as result of the discovery of their roles in molecular recognition, transmembrane signaling and other physiological or pathological processes. Therefore the applications of oligosaccharides as pharmaceuticals in the fields of the prevention of infections by pathogens, neutralisation of toxins, regulations of inflammation and cancer immunotherapy have been widely recognised [74].

An important factor limiting the industrial application of enzymes is the ability to carry out cost-effective cofactor recycling, particularly those involving carbon-carbon formation. Leloir glycosyltransferase catalysis, that attracted significant research attention for well over 30–40 years, is one of the common ways to synthesise oligosaccharides. Thus, it is dependent on the availability of expensive sugar nucleotides. Therefore, in the last two decades, numerous studies were conducted to develop techniques for large-scale synthesis of oligosaccharides by the use of plant and bacterial activities. The progress of the biotechnological sector enabled the construction of genetically engineered microorganisms which allowed for example the mass production of glycosyltransferases for enzymatic synthesis of oligosaccharides [75].

Thus most commercial enzymes are hydrolases and isomerases. Basic and applied biotechnological research enlarges continuously the diversity of enzymes suitable for industrial purposes. Among the actual “trendy” enzymes, the polygalacturonases and transferases represent excellent candidates for selectively catalysing high-yielding synthetic reactions under mild conditions.

Up to now, polygalacturonases have found application in food industry by the clarifying of soft-drinks (from fruits and vegetables) and wine [34]. Alternatively, recombinant polygalacturonases can successfully catalyse transglycosylation reactions for the synthesis of new compounds [76].

Especially the transferases, which constitute over 25% of known enzymes, stand in the spotlight of latest research because of their functional properties. Particularly the fructosyltransferases may be good applicants for the next generation of industrial catalysts, being able to fulfil actual and prospected market trends, since they initiate innovative routes for the synthesis of novel oligosaccharides [77].

Enzymatic oligosaccharide synthetic pathways using the exo-polygalacturonase and FTF, which were studied in this thesis, are able to overcome the difficulties associated either with chemical methods or, for FTF, with biocatalytic Leloir synthesis. The two enzymes offer attractive operational routes with few steps from substrate to product, although occur with high yields, time- and cost-effectiveness. As substrates for these industrial feasible pathways, cheap renewable materials such as sugar-beet pulp and sucrose can be successfully used.

Products as sucrose analogues are low in cariogenity and sweetness, making them of interest as sucrose replacements in areas where excessive sweetness is a problem. Galactosucrose (10-15% sweetness compared to sucrose) is interesting both due to its prebiotic role and because it may be produced also from molasses [44].

Furthermore, Gal-Fru synthesised by the FTF from *B. subtilis* NCIMB 11871 can be used in a novel approach to define the specificity of native and modified glucosyltransferases, respectively dextransucrases and to extend their substrate spectrum.

Sucrose, also called “royal carbohydrate”, represents the most abundantly produced renewable organic compound (world production 120 million tons/year) and this in unequalled purity and to an exceptionally low price. Until now, only 3% of the sucrose global production finds application in non-food sectors. Therefore, the intended replacement of fossil raw materials by those annually regrowing requires systematic basic researches in the entire spectrum of promising applications, in order to investigate and improve their competitiveness over petrochemical raw materials [78].

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APPENDIX

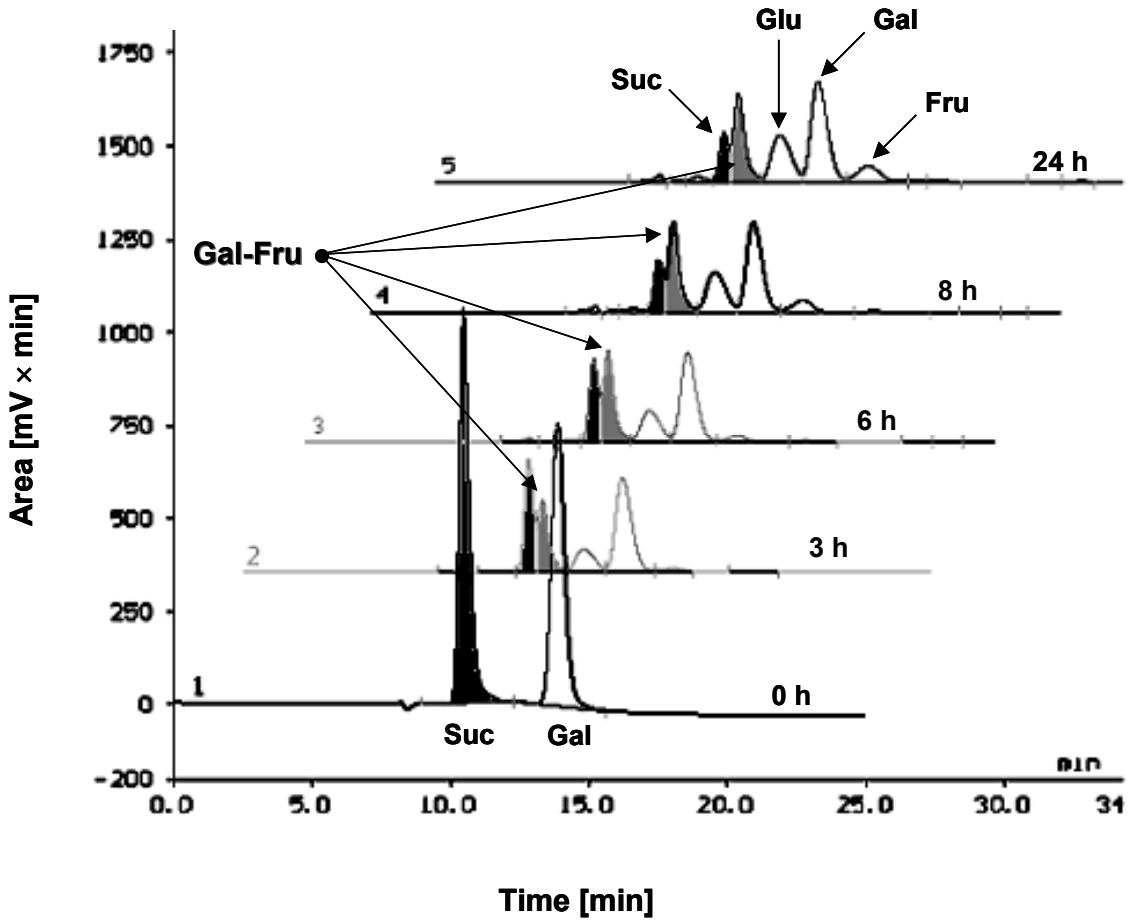

Fig. 51: Gal-Fru synthesis with FTF supernatant

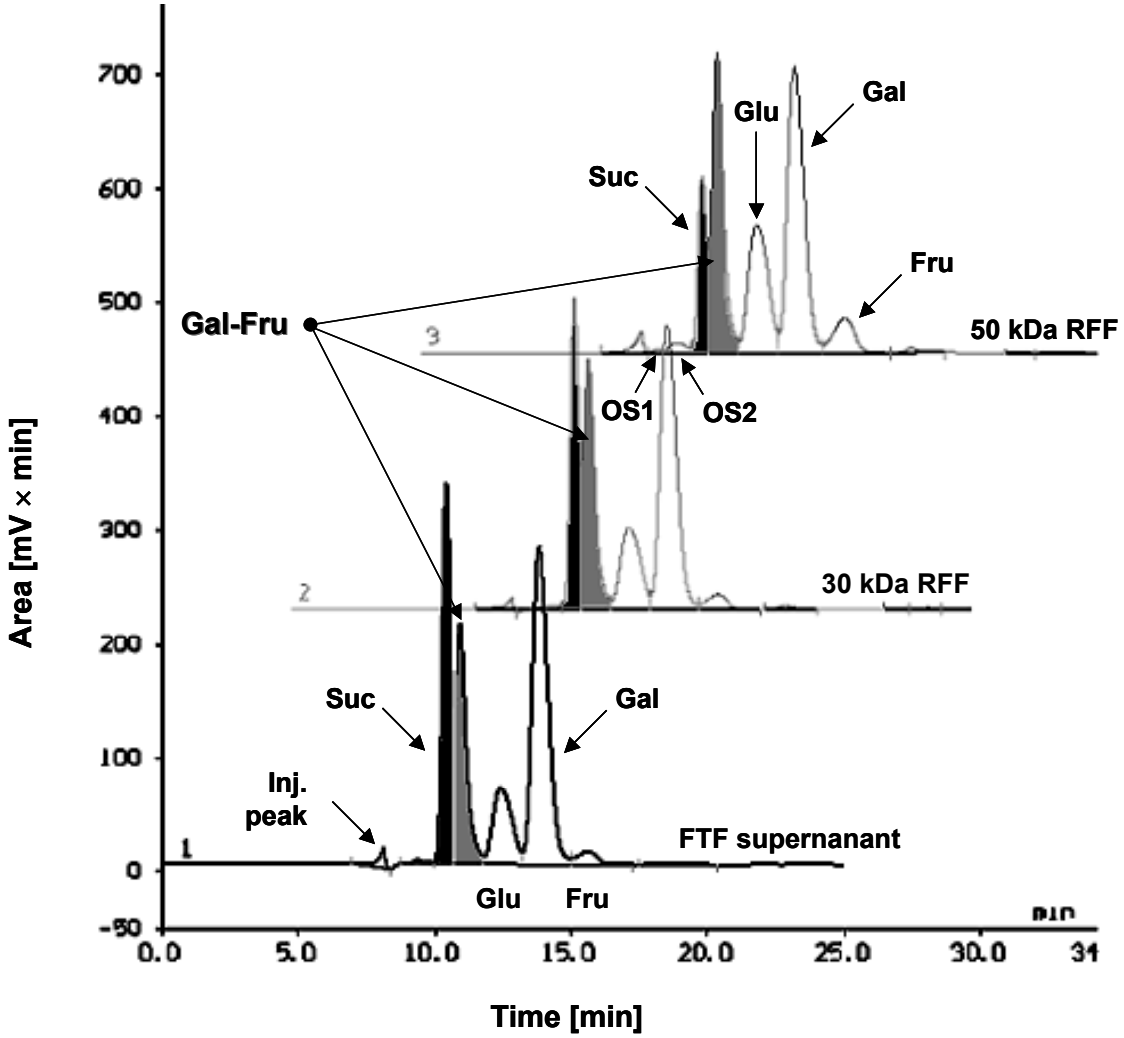

Fig. 52: Gal-Fru synthesis with FTF supernatant, 30 kDa RFF and 50 kDa RFF: after 6 h

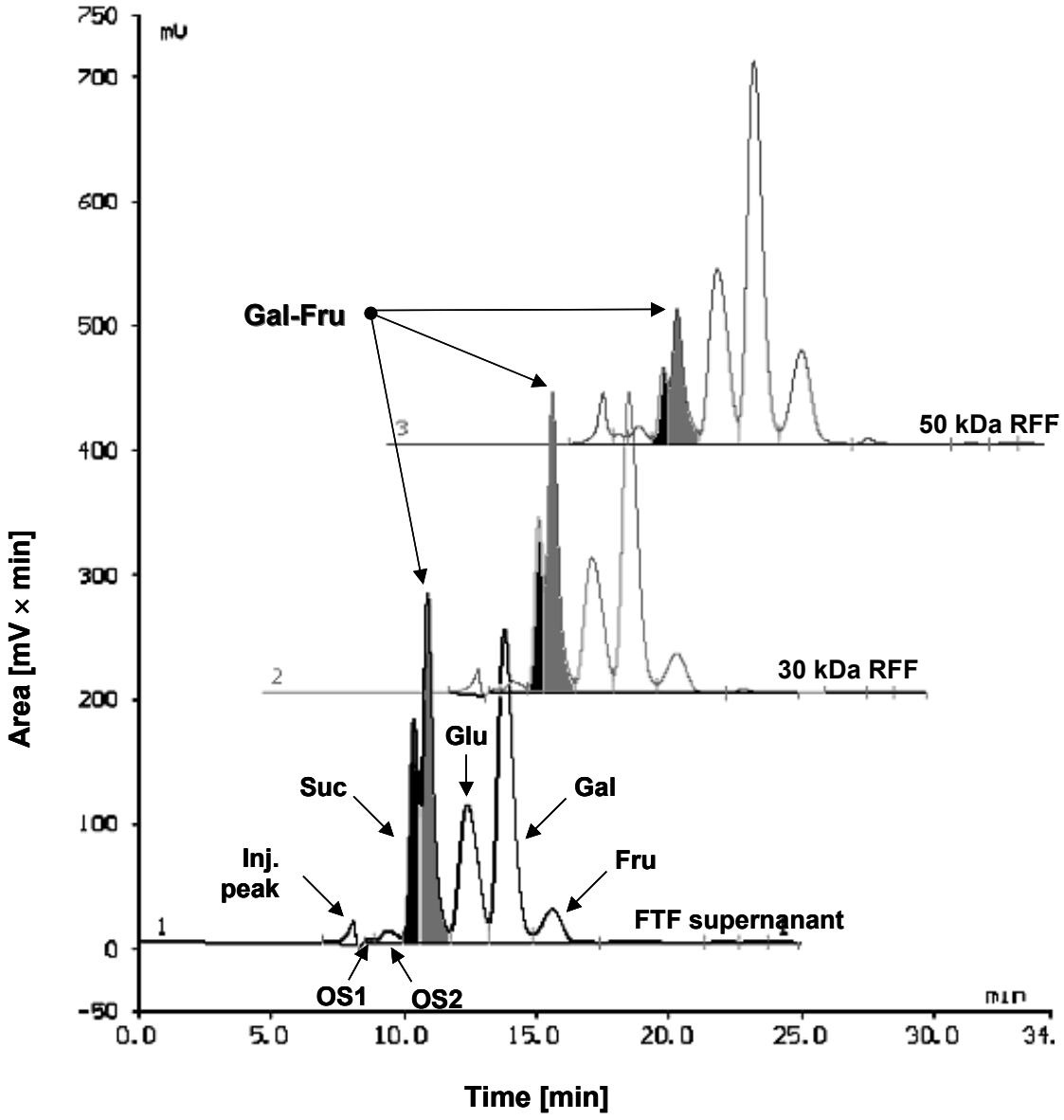

Fig. 53: Gal-Fru synthesis with FTF supernatant, 30 kDa RFF and 50 kDa RFF: after 24 h

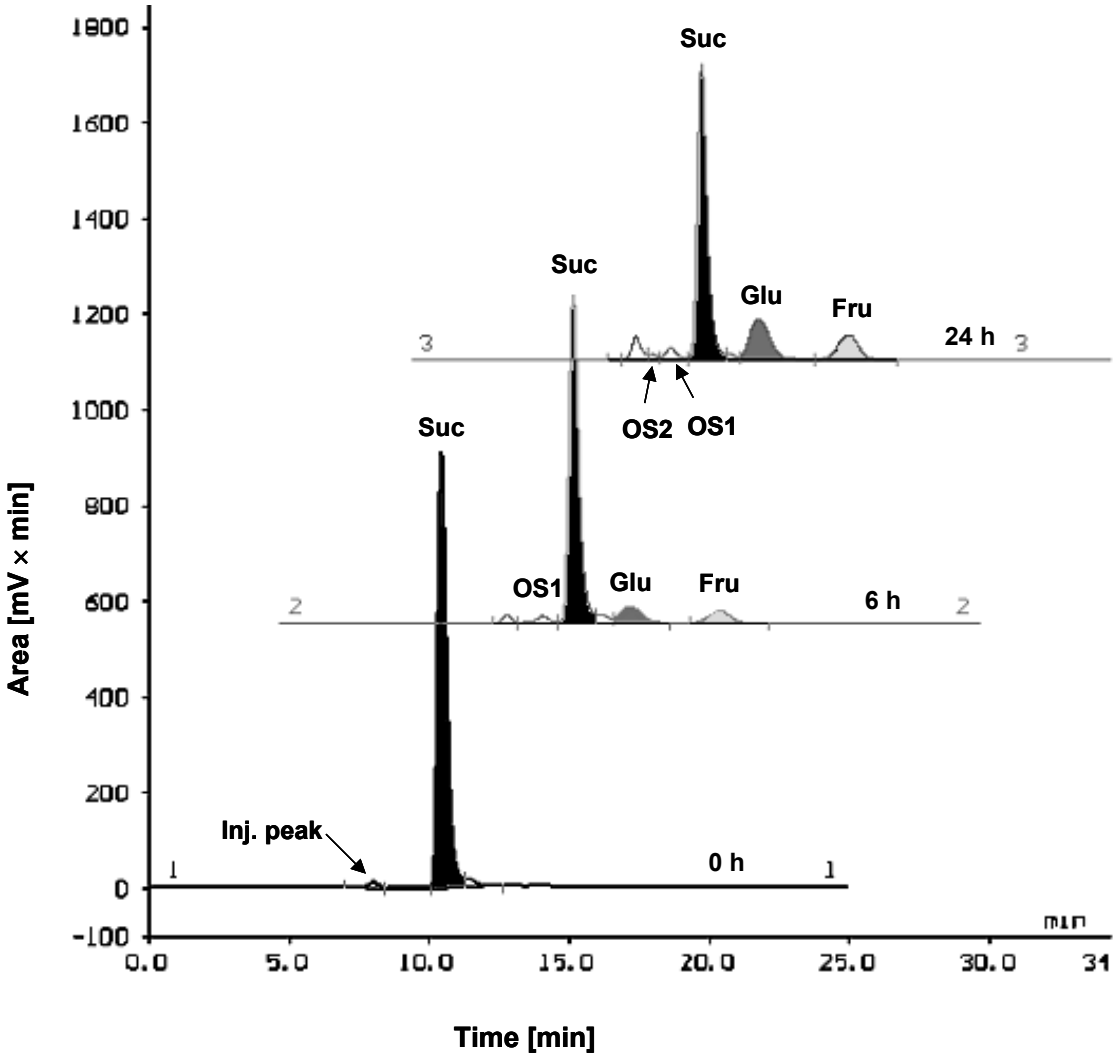

Fig. 54: Sucrose hydrolysis with FTF supernatant

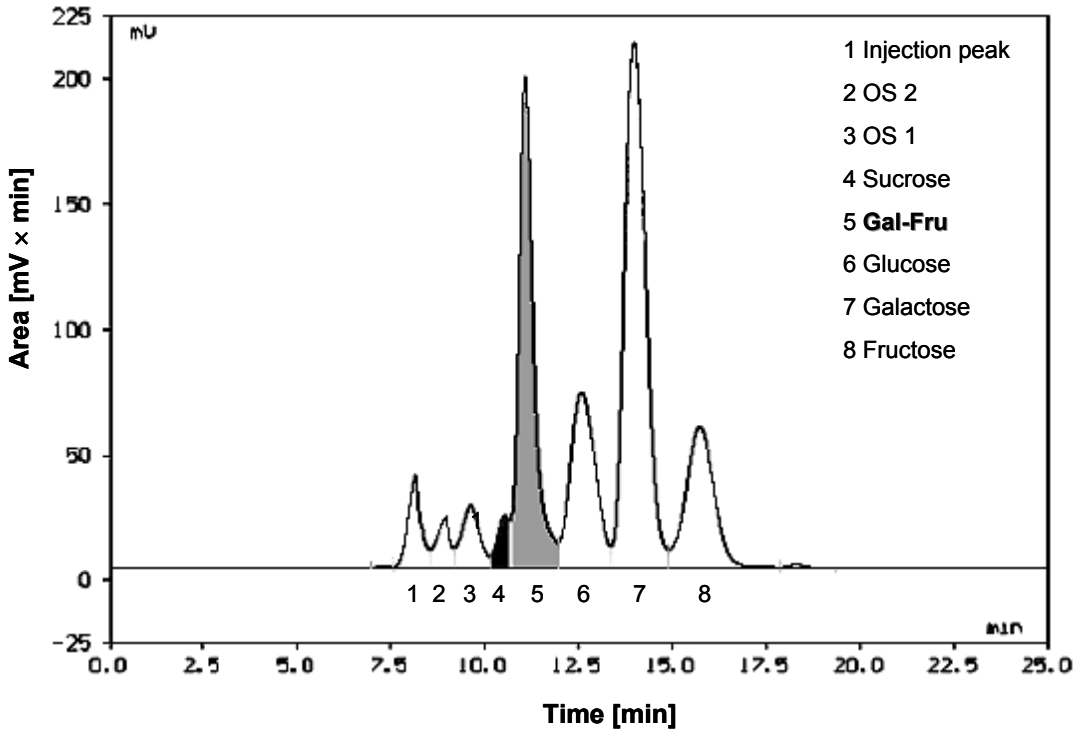

Fig. 55: Preparative chromatographic separation with PCR 6 Na^+ of a sample obtained by treatment of the Gal-Fru substrate solution with immobilised DS/α-glucosidase (chromatogram related to Tab. 21)

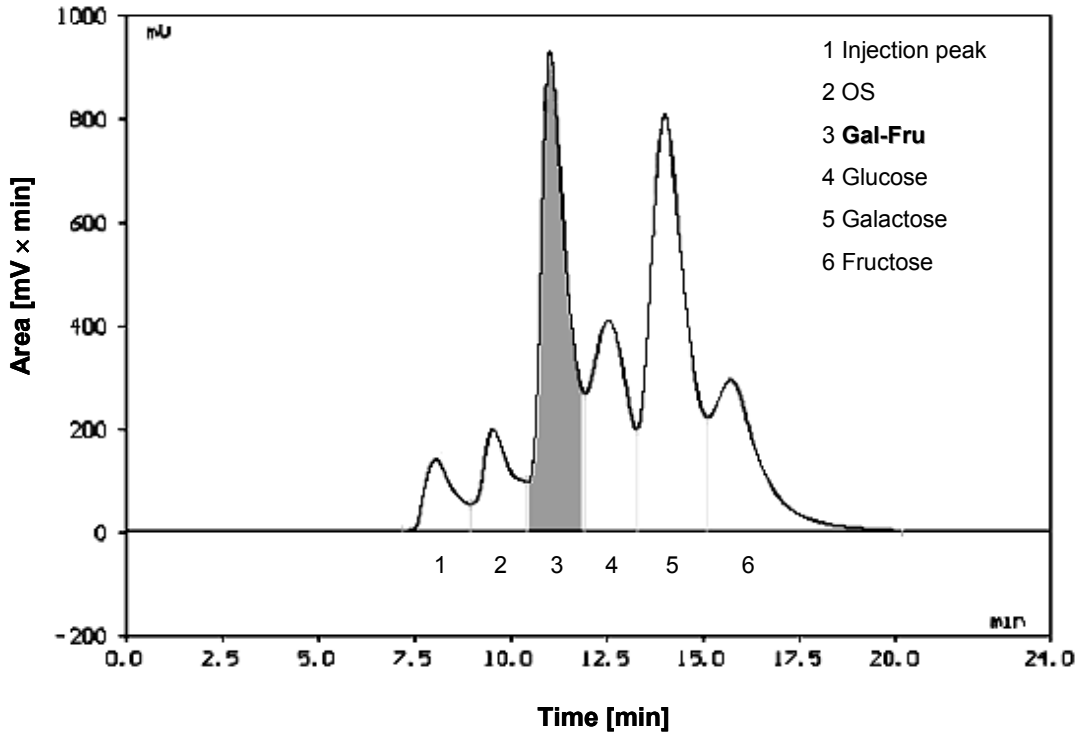

Fig. 56: Preparative chromatographic separation with PCR 6 Na^+ of a sample obtained by treatment of the Gal-Fru substrate solution with DS from *S. oralis* (chromatogram related to Tab. 22)

Tab. 25: Preparative chromatographic separation on PCR 6 Na^+ of the carbohydrates mixture after the treatment of the Gal-Fru substrate solution with immobilised DS/α-glucosidase

Fraction Nr.	Flow [mL/min]	Vol.[mL]	OS	Suc	Gal-Fru	Glu	Gal	Fru	Total area	[Gal-Fru]/ [Total] [%]	[Gal-Fru area]/ [Suc area]
Dead vol.	4.5	670.0	-	-	-	-	-	-	0.0	-	-
1	4.0	682.0	0.8	-	-	-	-	-	0.8	-	-
3	4.5	707.5	149.1	-	-	-	-	-	149.1	-	-
5	4.2	732.0	360.0	-	3.8	-	-	-	363.9	1.1	-
7	4.0	756.5	328.8	-	5.8	-	-	-	334.6	1.7	-
9	4.2	781.0	160.9	-	3.9	-	-	-	164.8	2.3	-
11	4.2	806.5	145.4	-	2.4	-	-	-	147.8	1.6	-
13	4.2	830.5	133.9	-	1.1	-	-	-	135.0	0.8	-
15	4.2	855.0	127.9	-	3.1	-	-	-	131.0	2.4	-
17	4.2	879.5	117.6	-	3.8	-	-	-	121.4	3.1	-
19	4.3	904.5	117.3	-	14.0	-	-	-	131.3	10.6	-
21	4.2	929.0	83.8	11.2	57.3	-	-	-	141.1	40.6	5.1
23	4.2	954.0	61.3	21.8	161.0	-	-	-	222.3	72.4	7.4
25	**4.3**	**979.0**	**26.7**	**36.5**	**357.6**	**-**	**3.8**	**2.8**	**427.3**	**83.7**	**9.8**
27	**3.8**	**1002.5**	**3.5**	**39.9**	**506.0**	**-**	**4.0**	**3.4**	**556.7**	**90.9**	**12.7**
29	**4.2**	**1027.5**	**0.6**	**25.5**	**406.5**	**28.3**	**4.7**	**3.7**	**469.4**	**86.6**	**15.9**
31	**4.0**	**1052.5**	**-**	**12.1**	**203.1**	**27.3**	**5.8**	**4.6**	**252.8**	**80.3**	**16.9**
33	4.2	1077.0	-	4.9	102.6	25.1	7.5	4.7	144.8	70.9	20.7

Fraction Nr.	Flow [mL/min]	Vol.[mL]	OS	Suc	Gal-Fru	Glu	Gal	Fru	Total area	[Gal-Fru]/ [Total] [%]	[Gal-Fru area]/ [Suc area]
35	3.8	1101.0	-	4.2	69.3	89.6	16.5	5.7	185.3	37.4	16.6
37	3.8	1124.0	-	4.0	45.1	172.2	59.7	19.2	300.3	15.0	11.2
39	4.0	1148.0	-	-	10.5	302.8	224.9	70.3	608.5	1.7	-
41	4.2	1172.5	-	-	4.3	337.9	482.1	174.1	998.4	0.4	-
43	3.8	1196.5	-	-	3.2	291.6	686.4	276.0	1257.2	0.3	-
45	4.0	1220.5	-	-	2.8	166.9	808.7	358.9	1337.3	0.2	-
47	4.0	1244.5	-	-	2.3	53.4	741.0	386.0	1182.7	0.2	-
49	4.0	1269.0	-	-	1.9	19.7	436.7	276.3	734.6	0.3	-
51	4.0	1293.5	-	-	1.7	10.9	201.2	140.9	354.7	0.5	-
53	4.2	1318.0	-	-	1.4	8.9	138.6	96.6	245.5	0.6	-
55	4.0	1342.5	-	-	0.7	6.8	80.6	51.4	139.5	0.5	-
57	4.2	1367.0	-	-	-	5.3	52.2	31.1	88.6	-	-
59	4.3	1392.0	-	-	-	9.8	36.7	21.1	67.6	-	-
61	4.2	1418.0	-	-	-	2.7	25.1	13.2	41.0	-	-
63	3.7	1441.0	-	-	-	2.2	17.1	8.8	28.1	-	-
65	4.2	1453.5	-	-	-	1.9	12.7	6.5	21.2	-	-
67	4.3	1465.5	-	-	-	0.5	6.9	4.0	11.4	-	-
69	4.2	1478.0	-	-	-	0.5	5.0	2.8	8.2	-	-
71	3.7	1489.0	-	-	-	0.5	4.3	2.3	7.1	-	-

Tab. 26: Preparative chromatographic separation on PCR 6 Na^+ of the carbohydrates mixture after the treatment of the Gal-Fru substrate solution with *S. oralis* dextransucrase

Fraction Nr.	Flow [mL/min]	Vol. [mL]	OS 1	OS 2	Gal-Fru	Glu	Gal	Fru	Total	[Gal-Fru]/ [Total] [%]
Dead vol.	5.0	690.0	2.4	-	-	-	-	-	2.4	-
2	4.0	719.0	6.5	-	-	-	-	-	6.5	-
4	4.7	745.0	12.4	-	-	-	-	-	12.4	-
6	5.0	773.0	29.4	8.2	-	-	-	-	37.6	-
8	4.2	798.0	94.3	19.0	1.8	-	-	-	115.1	1.5
10	3.7	820.5	134.0	37.2	3.1	-	-	-	174.3	1.8
12	4.0	844.5	124.7	48.3	5.8	-	-	-	178.8	3.3
14	4.0	868.5	82.1	57.7	9.4	-	-	-	149.2	6.3
16	4.2	893.0	41.7	64.1	21.6	-	-	-	127.3	17.0
18	4.2	917.5	24.0	75.6	43.6	-	-	-	143.2	30.5
20	4.0	942.0	13.4	97.4	88.3	-	-	-	199.1	44.4
22	4.3	967.5	6.8	86.7	174.8	-	-	-	268.2	65.2
24	**4.2**	**992.5**	**2.1**	**62.3**	**330.6**	**-**	**-**	**-**	**395.0**	**83.7**
26	**4.0**	**1016.5**	**-**	**36.2**	**462.0**	**-**	**-**	**-**	**498.3**	**92.7**
28	**4.2**	**1041.0**	**-**	**18.0**	**528.7**	**-**	**-**	**3.6**	**550.3**	**96.1**
30	**3.7**	**1064.5**	**-**	**8.0**	**436.1**	**12.8**	**3.3**	**4.2**	**464.5**	**93.9**
32	**4.2**	**1088.5**	**-**	**4.0**	**232.4**	**25.0**	**4.4**	**4.6**	**270.4**	**85.9**
34	5.0	1118.5	-	2.3	148.0	45.9	6.1	5.3	207.5	71.3

Fraction Nr.	Flow [mL/min]	Vol. [mL]	OS 1	OS 2	Gal-Fru	Glu	Gal	Fru	Total	[Gal-Fru]/ [Total] [%]
36	5.2	1150.0	-	1.1	71.7	87.2	15.1	12.6	187.8	38.2
38	5.2	1181.0	-	-	21.7	169.9	58.4	21.0	271.0	8.0
40	5.2	1212.5	-	-	10.2	297.6	120.9	48.4	477.1	2.1
42	5.0	1244.0	-	-	7.4	335.6	247.6	82.5	673.1	1.1
44	5.5	1276.5	-	-	5.7	289.3	477.7	163.2	935.9	0.6
46	5.0	1308.0	-	-	2.9	164.6	664.0	235.5	1067.0	0.3
48	5.2	1339.5	-	-	2.0	51.1	791.3	327.7	1172.0	0.2
50	4.8	1370.0	-	-	1.9	17.4	712.4	364.6	1096.3	0.2
52	5.0	1400.5	-	-	1.7	8.6	509.4	265.1	784.8	0.2
54	4.8	1430.0	-	-	1.4	6.5	233.5	136.0	377.5	0.4
56	5.3	1462.0	-	-	1.2	4.5	125.3	94.1	225.0	0.5
58	5.2	1493.5	-	-	0.8	2.9	79.2	48.9	131.9	0.6
60	5.0	1524.0	-	-	0.6	7.5	50.8	28.3	87.3	0.7
62	4.0	1548.0	-	-	0.2	3.5	35.3	21.6	60.7	0.4
64	4.2	1573.0	-	-	0.1	1.1	23.7	11.8	36.9	0.4
66	4.0	1596.0	-	-	-	0.5	15.8	7.4	23.7	-
68	4.3	1620.0	-	-	-	-	11.4	5.2	16.6	-
70	4.0	1644.0	-	-	-	-	5.5	3.8	9.2	-
72	4.0	1669.0	-	-	-	-	3.6	2.4	6.0	-
74	4.0	1719.0	-	-	-	-	1.1	0.9	2.0	-
76	4.0	1744.0	-	-	-	-	0.7	0.5	1.3	-
78	4.0	1768.0	-	-	-	-	0.4	0.2	0.6	-

FIGURES INDEX

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